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Thyroid hormone signalling in Osteoarthritis: early life events in late life disease

Bömer, N.

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Thyroid hormone signalling in Osteoarthritis

Early life events in late life disease

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Thyroid hormone signalling in Osteoarthritis; Early life events in late life disease

N. Bömer, MSc

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Early life events in late life disease

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Promotor

Prof. dr. P.E. Slagboom

Co-promotor

Dr. I. Meulenbelt

Leden promotiecommissie

Prof. Dr. R.G.H.H. Nelissen

Prof. Dr. H.B.J. Karperien

Department of Developmental BioEngineering; University of Twente

Dr. E.N. Blaney-Davidson

Department of Rheumatology; Radboud University Medical Center

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Chapter 1

General introduction

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Introduction

Osteoarthritis (OA) is the most common arthritic disorder and is known as an age-related, prevalent, complex, chronic and disabling disease of articular joints. Disease progression is characterized by progressive destruction of joint articular cartilage, remodeling of the subchondral bone, formation of osteophytes and synovitis^{1,2}. Structural changes to OA cartilage include aberrant extracellular matrix (ECM) content and surface disruptions that range from fibrillation, clefting and delamination to complete articular surface erosion³⁻⁵. The complex nature of the disorder is likely based on interindividual variation in genetics, set-points in development, joint geometry and repair capacity. These inherited differences between individuals likely modulate the effects of secondary factors ranging from mechanical (over)loading^{6,7} and BMI^{8,9} to systemic inflammation^{10,11}.

Currently, there is no adequate therapy to reverse or slow down the disease. Since over 20% of the Dutch population is OA affected, of which 80% have limitations in movement and 25% inhibition in major daily activities of life, OA puts a high social and economic burden on society. In the Netherlands the total costs for OA were estimated to be €1.1 billion in 2011, equivalent to 1.2% of the gross national health care costs (National Compass The Netherlands, 2011). Even more, OA is distinguished by its growing prevalence due to increasing life expectancy and the obesity epidemic. According to the United Nations, by 2050 people aged over 60 will account for more than 20% of the world's population.¹² This means that by 2050, 130 million people will suffer from OA worldwide, of whom 40 million will be severely disabled by the disease.¹²

Research into causal factors inducing OA focuses on genetics and cell-biological approaches. Comprehensive genome wide searches for genetic variants conferring risk for OA have resulted in robust genome wide significant signals.^{13,14} Functional follow up studies, to show biological relevance of the loci identified, have only recently started to be implemented as a logical next step. Consequently, little progress has been made in clinical translation of these findings, let alone identification of novel evidence based treatment options or disease modifying OA drugs. A more systematic dissection of underlying biological mechanisms of discovered OA susceptibility genes will deepen our fundamental understanding of the different OA disease pathways and respective disease drivers.

In this introduction we outline the disease processes and mechanisms that we face in OA research. We focus particularly on the role of local thyroid hormone signalling, and discuss which steps should be taken to go from a genetic association to functional genomics analyses in such a way that we obtain mechanistic understanding of genetic variation, gene expression and epigenetics underlying OA susceptibility. By subsequently finding ways to counteract identified aberrant disease processes, evidence based treatment options should be identified.

Articular cartilage

Articular cartilage is the connective tissue that covers the end of long bones. It has a smooth, wear-resistant lubricated surface that allows bones to glide over one another with minimal friction, yet eligible to absorb impact forces. Cartilage consists of only one cell type embedded in an extensive network of ECM and does not have nerves, blood vessels, or a lymphatic system¹⁵. The single cell type present in cartilage is the articular chondrocyte, yet contributes only ~5% to the total cartilage volume. Articular chondrocytes reside in a maturational arrested state without detectable proliferation and at a low metabolic activity.¹⁶ Nonetheless, chondrocytes maintain cartilage tissue homeostasis throughout life and, as such, they need to become metabolically active and apply remodelling of the extracellular matrix upon micro-traumas.¹⁷ To cope with these challenges, the chondrocyte is required to continuously and dynamically adjust expression of catabolic and anabolic genes, while securing its capacity to restore its maturational arrested steady state phenotype.¹⁸ In OA affected cartilage, articular chondrocytes have seemingly lost their maturational arrested state as they proliferate and get a growth plate chondrocyte-like morphology, similarly as during endochondral ossification¹⁹, thereby exhibiting articular cartilage debilitating activity.²⁰ Endochondral ossification is the developmental process of bone formation in which mesenchymal stem cells condensate and subsequently differentiate into chondroblasts in order to establish the cartilage anlage; a matrix rich in type II collagen and specific proteoglycans such as aggrecan²¹. The chondroblasts then become chondrocytes and organize into zones that form the growth plates of bone. The chondrocytes become hypertrophic and commence terminal differentiation, followed by mineralization of the cartilage anlage, apoptosis of the chondrocytes and vascular invasion¹⁹. In this process, local, intracellular active thyroid hormone (T3) is known to be essential in signalling terminal maturation of hypertrophic chondrocytes²².

Disease mechanisms

As much as endochondral ossification is essential during skeletal development, as deleterious it is when taking place in mature articular cartilage ([Figure 1](#)).

Genetic susceptibility to OA

OA has a considerable, but complex, genetic component²⁴; many genetic variants (SNPs) with small effects are expected to influence the onset and course of the disease.^{24, 25} Genetic studies in OA originally included mainly candidate gene association and genome wide linkage studies, yet extended in more recent years to large scale genome wide association studies (GWAS), including large case/control studies. Based on genome wide significance ($P < 5e-8$) and/or compelling functional follow up, these studies have resulted in a list of acknowledged susceptibility loci²⁶⁻²⁹.

Despite the phenotypic complexity of OA, robust genetic evidence was produced for a common underlying mechanism involving the maturational process of growth plate chon-

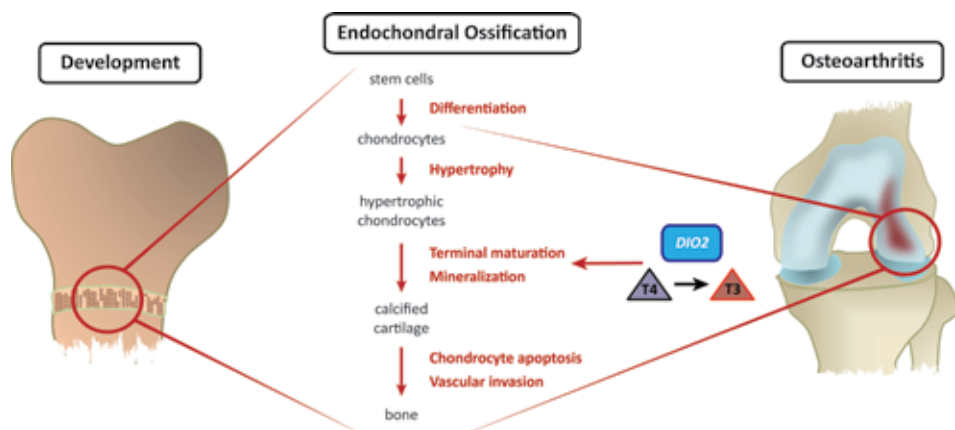


Figure 1. Reoccurrence of developmental mechanisms in OA pathophysiology.

Growth plate chondrocyte maturation in the process of endochondral ossification is essential during skeletal development (left). The occurrence of this process in adult articular cartilage forces maturational arrested chondrocytes to commence hypertrophic differentiation and terminal differentiation (right). The bio-availability of intracellular active thyroid hormone (T3) is known to be essential in this signaling cascade and recuperation of this growth plate morphology and signaling of articular chondrocytes is recognized as a hallmark of end stage OA pathophysiology²³.

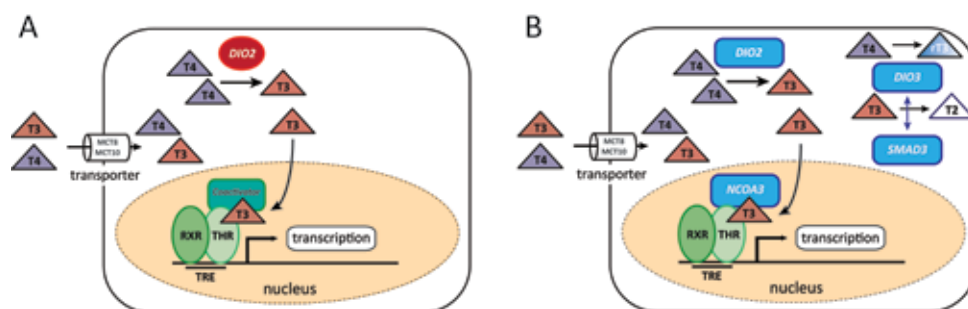


Figure 2. Graphical representation of the processes involved in intracellular thyroid hormone availability.

(A) Thyroid hormone responsive cells, like chondrocytes, carry cell membrane transporters such as the monocarboxylate transporters 8 and 10 (*MCT8*, *MCT10*) admitting inactive (T4) and active (T3) thyroid hormone⁴⁷. Intracellular 3,3',5,5'-tetraiodothyronine (T4) can subsequently be activated into 3,3',5-triiodothyronine (T3) by iodothyronine-deiodinase enzyme type 2 protein (D2)⁴⁸, encoded by *DIO2*²⁸. Thereby, D2 regulates the local T3 bio-availability in specific target tissues such as the growth plate, but not systemically⁴⁸. After entering the nucleus, T3 binds Thyroid Hormone Receptors (THRs). In general, THRs are located in the nucleus, where they homo- or hetero-dimerize with another nuclear receptor, predominantly RXR, before activating target gene expression. The formed dimers bind thyroid hormone response elements (TRE) in the promoters of target genes and recruit co-activators to start gene transcription upon T3-binding⁴⁹. (B) Inclusion of the OA associated genes connected to thyroid hormone signalling (*NCOA3*, *DIO3* and *SMAD3*).

drocytes which coordinates the formation of cartilage and bone during endochondral ossification.³⁰⁻³⁵ But how do the OA susceptibility genes, known for coordinating early life events, influence the onset of OA at a later stage in life? Our group proposed, 7 years ago, two possible hypothesis regarding this question. One hypothesis was that the susceptibility genes influence early suboptimal skeletal morphogenesis, thereby increasing OA susceptibility. The second hypothesis was that the genes contribute to chondrocyte dedifferentiation later in life as a consequence of aging^{36, 37}.

In order to answer these hypotheses, translation of these gene deviations towards underlying biological mechanisms is necessary. Nevertheless, the number of published genetic studies that lack experimental data showing the biological relevance of identified genetic variation is still large, it was advocated that enhanced implementation of functional genomics is needed to substantially augment translation to drug development and disease management²⁶.

DIO2 susceptibility to OA

By applying genome wide linkage analyses and replication by association to symptomatic OA, the deiodinase iodothyronine type-2 (D2) gene (*DIO2*)³⁸ was identified by our group as an OA susceptibility gene. The finding comprised consistent associated risk of the C-allele of the nonsynonymous rs225014 single nucleotide polymorphism (SNP) located in the coding region of *DIO2*³⁸. Multiple follow up studies comparing OA affected and healthy articular cartilage in both mice³⁹ and humans⁴⁰⁻⁴² demonstrated significant upregulation of *DIO2* expression in OA affected cartilage, as well as a marked upregulation of D2 protein that positively correlated with increasing Mankin-score⁴³, a histology based marker of OA severity. It was investigated whether the *DIO2* risk alleles confer risk to OA via non-optimal shape of the bones in the joint consequently leading to recurrent damage of the cartilage and eventually triggering the OA onset^{44, 45}. With respect to skeletal formation it was shown that the *DIO2* OA risk allele did not directly affect hip geometry. More likely, the cartilage structure or metabolism was affected leading to OA susceptibility as result of a non-optimal hip morphology and respectively induced biomechanical stresses⁴⁶.

Thyroid hormone and OA; a more common OA

Identification of *DIO2* as OA-susceptibility gene and the presence of *DIO2* mRNA expression and D2 protein in articular cartilage pointed at the importance of local thyroid hormone availability in the aetiology of symptomatic OA. The identified *DIO2* gene encodes an enzyme that regulates intracellular bioavailability of active thyroid hormone T3 ([Figure 2A](#)).

In thyroid hormone responsive cells like growth plate chondrocytes, T3 initiates the coordinated progression of endochondral ossification during pre-natal development and post-natal linear growth, facilitating the terminal maturation of hypertrophic chondrocytes^{22, 50-52}. As mentioned before, there are striking parallels between chondrocyte signalling occurring in the downstream events of the growth plate and the behaviour of chondrocytes in OA affected articular cartilage ([Figure 1](#))²³. The relevance of intracellular thyroid signaling for the onset/development of OA was underscored by the suggested

modulation of osteoarthritis disease risk by the *DIO3* gene⁵³. Candidate gene association studies indicated the protective effect of the G-variant of the rs945006 SNP located in the 3'-UTR of the deiodinase iodothyronine type-3 (D3) gene (*DIO3*)⁵³. D3 enzymatically generates inactive metabolites (rT3 and T2) by removing a 5-iodine atom from T4 or T3, thereby diminishing levels of active T3^{48, 54} and together with its counterpart D2, providing an elegant homeostatic mechanism to ensure intracellular T3 bio-availability locally, not systemically^{48, 54}. A broader network of genes, involved in T3-signalling, confer OA-susceptibility by reversing T3 bio-availability (*DIO3*)^{50, 53}, influencing the T3 bio-availability machinery (*SMAD3*)^{55, 56} and by transcriptional co-activation of thyroid responsive genes under influence of T3 (*NCOA3*)^{57, 58} (Figure 2B). However, to understand how increased *DIO2* expression/T3 bio-availability affects articular cartilage and its relevance for the onset/development of OA we needed to commence functional analyses.

Methodology: studies and approaches used in this thesis

To comprehend how causality is explained by genetic variants, such as for *DIO2*, we need to understand the molecular consequences of these variants for gene expression and epigenetic regulation. Besides the study of disease-relevant tissues, which will contribute to a deeper insight into ongoing pathophysiological processes as result of the gene deviations, the study of respective cells will be key to establish *in vitro* models to further characterize genetic variants at a transcriptional and functional level and ultimately the use of an *in vivo* model organism will be needed.

RAAK-Biobank

Of great value to reach this aim is the availability of the RAAK-biobank, in which multiple disease relevant tissues (OA affected- and macroscopically unaffected cartilage, bone and ligament) and cells (bonemarrow-derived mesenchymal stem cells (hBM-MSCs) and primary articular chondrocytes (hPACs)) were collected from patients (N=400) who underwent total joint arthroplasty as result of end stage OA at the LUMC⁵⁹. A disease-specific biobank is tailored for dissecting molecular mechanisms underlying disease onset.

Allelic Imbalanced Expression

A possible mechanism by which genetic variation could confer to OA susceptibility is the selective transcription of alleles in heterozygous carriers of disease associated SNPs⁶⁰. Such allelic imbalanced expression (AIE) as marked by the intragenic OA susceptibility SNP rs225014 could be assessed in *DIO2* mRNA in OA cartilage. The OA risk allele 'C' being approximately 1.3 times more abundantly present than the wild-type allele 'T' (Figure 3).

Regarding the hypothesis postulated in 2008, this subtle difference in allelic expression throughout life could be detrimental to the articular cartilage and may underlie OA disease onset and progression. AIE was investigated by several groups for multiple susceptibility loci, such as *GDF5*⁶¹, *SMAD3*⁶², *NCOA3*⁶³, *GNL3* and *SPCS1*⁶⁴, *COL11A1*⁶⁵ and

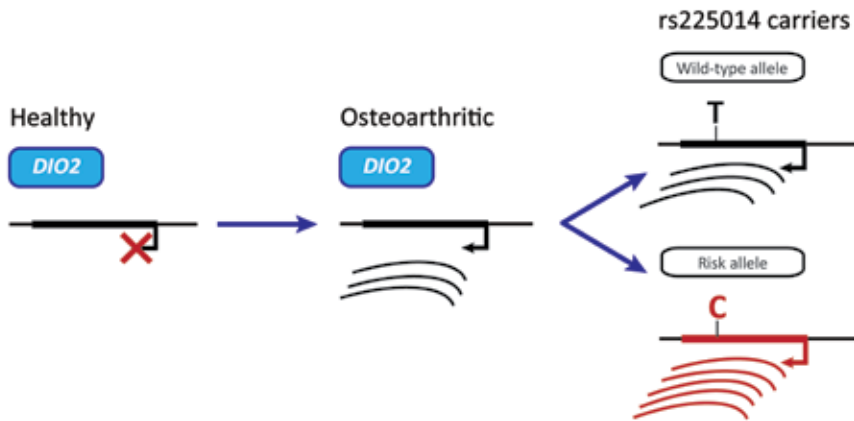


Figure 3. Graphical representation of the AIE mechanism of *DIO2* susceptibility.

Loss of silencing of *DIO2* expression may affect the propensity of maturational arrested chondrocytes to adopt an OA like state.

*DIO2*⁶⁶. Indeed for most of the loci, AIE of OA risk alleles associated to disease susceptibility⁶⁷, as is also thought true for complex traits in general.^{68,69}

Epigenetic regulation of gene expression

Epigenetics involves regulatory mechanisms that can switch genes on or off and determine which proteins are transcribed by factors other than an individual's DNA sequence. The most studied epigenetic mechanism is DNA methylation; the phenomenon in which the cytosine nucleotide that is located in cytosine-guanine (CpG) residue pairs is methylated. Genetic variations⁷⁰ and stochastic events due to injury or during ageing have been shown to cause changes in epigenetic marks resulting in subtle differential (allelic) gene expression. Moreover, such changes have frequently been found to be associated to pathological conditions⁷¹, including OA⁷². The effect of the *DIO2* risk allele on local DNA methylation and subsequent *DIO2* gene expression, however, remains elusive.

In vitro cell-models for OA research

Generally, the next step in translating disease susceptibility loci towards biological relevance is applying *in vitro* cell models especially suitable to investigate the consequences of genetic and transcriptional variation of candidate genes. Different *in vitro* models can be employed to investigate the effects on chondrogenesis. When utilizing human cells (hBM-MSCs or hPACs), 2 types of cell models are applied: 2D monolayer cultures and 3D micromass cultures. Comparing monolayer (2D) versus micromass (3D) cultures showed that the basal gene- and protein-expression in these cultures were fundamentally different⁷⁷. The use of monolayer cultures mainly resulted in complete loss of chondrogenic⁷⁸,⁷⁹ and recovery of hypertrophic phenotype of the chondrocytes⁷⁷. In particular 3D models have been shown to support chondrocyte differentiation and proliferation and to produce superior cartilage-like tissue with enhanced mechanical properties⁸⁰. The 3D model of

hBM-MSCs, was proven to resemble, at consecutive weeks, chondrogenic differentiation, recapitulation, hypertrophy and terminal maturation processes thereby providing an elegant model of OA development, mimicking the re-couperation of the (unfavourable) process of endochondral ossification⁸¹.

In vivo animal models for OA research

Animal models further improve our understanding of the molecular mechanisms underlying genetic association, driving the OA pathology⁸³. With respect to cartilage destruction, different mouse strains have been generated or reused for known OA susceptibility genes. *Gdf5* knockout mice were generated long before the identification of the *GDF5* susceptibility locus and were used subsequently to study the development of OA⁸⁴. The same holds for *Smad3*-deficient mice⁸⁵. An exception is the generation of *Frzb* knockout-mice^{86, 87} specific for OA purposes.

Dio2 knockout mice were readily available and used in studies regarding, thermogenesis in brown adipose tissue⁸⁸, pituitary resistance to T4⁸⁹ and insulin resistance and susceptibility to diet induced obesity⁹⁰. No experiments were applied however to study joint pathology in OA. The only animal-model in which the functional effects of aberrant *DIO2* signalling on articular cartilage was assessed, was performed on cartilage-specific human *DIO2* overexpressing (hD2Tg) rats⁷⁶. Upon submission to an injury-induced OA model (DMM), hD2Tg rats showed a significant higher level of cartilage damage as compared to their wild type littermates. These results further suggested that increased D2 activity may in fact be detrimental for articular cartilage maintenance. It would therefore be of great value to assess the effect of *Dio2* deficiency on cartilage in an *in vivo* OA model utilizing *Dio2* knockout-mice. Recently, the model of biomechanical burden by exercise was put forward to provide a more physiological model of disease induction and progression⁹¹.

Aims of the thesis

In this thesis, we set out to elucidate and characterize the pathophysiological processes that underlie the re-occurrence of developmental signalling in a late life disease; Osteoarthritis.

In [chapter 2](#), as part of a large international collaboration, we identified susceptibility loci for hand OA by GWAS and particularly provided functional evidence for OA susceptibility of the *ALDH1A2* gene.

In [chapter 3](#), functional genomics analyses, including disease specific tissues and the *in vitro* micromass chondrogenesis model of hBM-MSCs, were applied as a tool to identify the underlying molecular mechanism of genetic variation, gene expression and epigenetics of the *DIO2* gene in the development of OA.

The beneficial effect of inhibiting *DIO2* in the *in vitro* model as found in [chapter 3](#) was translated to a *Dio2*-knockout mouse model in [chapter 4](#), in collaboration with the Skeletal Biology and Engineering Research Centre (KU Leuven, Belgium). The effect of forced

exercise was assessed, in a treadmill running model of OA, on knee-joints in *Dio2*^{-/-}-mice and their wild-type littermates.

In [chapter 5](#), we focus on *Calreticulin* (*Calr*), the single transcript reaching the significance threshold when comparing cartilage of wild-type- and *Dio2* knockout-mice. In parallel to disease modifiers, there is a need for insight into intrinsic factors that enhance stability of healthy articular chondrocytes. We aimed to elucidate the effect of altered *Calr* expression on cartilage homeostasis, as a mechanism underlying OA onset and facilitating the detrimental effects of increasing *Dio2* expression.

Finally, in [chapter 6](#), we focussed on the epigenetic landscapes of our *in vitro* micro-mass chondrogenesis models of hBM-MSCs and hPACs as compared to paired autologous articular cartilage, to get better understanding of the cell-type specific methylome for future OA research.

[Chapter 7](#) provides a general discussion, in which the results and future perspectives are discussed.

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Chapter 2

Severe osteoarthritis of the hand associates with markers within the *ALDH1A2* gene and with very rare variants at 1p31

Unnur Styrkarsdottir¹, Gudmar Thorleifsson¹, Hafþís Helgadóttir¹, Nils Bomer², Sarah Metrustry³, S. Bierma-Zeinstra⁴, Marian Beekman^{2,5,6}, Evangelos Evangelou⁷, Deborah Hart³, Annelieke M. Strijbosch², Aslaug Jonasdottir¹, Asgeir Sigurdsson¹, Finnur F Eiriksson⁸, Margret Thorsteinsdottir^{8,9}, Michael L. Frigge¹, Augustine Kong¹, Sigurjon A. Gudjonsson¹, Olafur T. Magnusson¹, Gisli Masson¹, The TREAT-OA consortium, arcOGEN consortium, Bert Hofman¹⁰, Þorvaldur Ingvarsson¹¹, Stefan Lohmander¹², Margreet Kloppenburg¹³, Fernando Rivadeneira^{4,10}, Rob G H H Nelissen¹⁴, Tim Spector³, Andre Uitterlinden^{4,10}, P Eline Slagboom^{2,5,6}, Ana M Valdes^{3,15}, Ingrid Meulenbelt^{2,6}, Joyce Van Meurs⁴, Unnur Thorsteinsdottir^{1,9}, Ingileif Jonsdottir^{1,9}, Helgi Jonsson^{9,16}, Kari Stefansson^{1,9}

¹deCODE Genetics/Amgen, Sturlugata 8, Reykjavik, Iceland. ²Dept. Molecular Epidemiology, Leiden University Medical Center, Leiden, The Netherlands. ³Dept of Twin Research, King's College London, St Thomas' Hospital, London, United Kingdom. ⁴Department of Internal Medicine, Erasmus Medical Center, Rotterdam, The Netherlands. ⁵IDEAL The Netherlands. ⁶Genomics Initiative, sponsored by the NCHA, Leiden, The Netherlands. ⁷Department of Hygiene and Epidemiology, University of Ioannina Medical School, Ioannina, Greece. ⁸Arctic Mass, Sturlugata 8, Reykjavik, Iceland. ⁹University of Iceland, Faculty of Medicine, Reykjavik, Iceland. ¹⁰Department of Epidemiology, Erasmus Medical Center, Rotterdam, The Netherlands. ¹¹Department of Orthopedic Surgery, Akureyri Hospital, Akureyri, Iceland and University of Akureyri, Institution of Health Science, Akureyri, Iceland. ¹²Department of Orthopedics, Clinical Sciences Lund, Lund University, Lund, Sweden. ¹³Dept. Clinical Epidemiology and Dept. Rheumatology, Leiden University Medical Center, Leiden, The Netherlands. ¹⁴Dept. of Orthopaedics, Leiden University Medical Center, Leiden, The Netherlands. ¹⁵Academic Rheumatology, University of Nottingham, Nottingham City Hospital, Nottingham, United Kingdom. ¹⁶Department of Medicine, Landspítali, The National University Hospital of Iceland, Reykjavik, Iceland.

Abstract

Osteoarthritis is the most common form of arthritis and a major cause of pain and disability in the elderly. Genome-wide association studies (GWAS) and meta-analyses efforts have yielded several significant loci for osteoarthritis of the hip and osteoarthritis of the knee¹⁻⁷. With the aim of discovering sequence variants that confer risk of osteoarthritis of the hand, we carried out a genome wide association study in subjects with severe hand osteoarthritis, using variants identified through a whole genome sequencing effort of 2,230 Icelanders. We found two significant loci in the Icelandic discovery set; at 15q22 (freq. 50.7%, OR 1.51, $P = 3.99 \times 10^{-10}$) in the *ALDH1A2* gene, and at 1p31 (freq. 0.02%, OR 50.6, $P = 9.8 \times 10^{-10}$). The rare variant at 1p31 segregates with osteoarthritis in one dense family, and is present in other populations. The markers within the *ALDH1A2* gene were confirmed in replication sets from the Netherlands and the United Kingdom, yielding an overall association of OR = 1.46 and $P = 1.1 \times 10^{-11}$ (rs3204689).

Osteoarthritis has a great impact on quality of life owing to pain and the loss of joint function. Osteoarthritis is characterized by a dynamic process involving the destruction and repair of joint tissues. It is a disease of the entire joint, affecting cartilage, synovium, subchondral bone, ligaments and the joint capsule¹. The clinical presentation of osteoarthritis is heterogeneous. Heritability estimates of osteoarthritis are in the range of 40–65%, depending on the joint affected^{2,3,4}. There is clear predilection for certain joints; the most common joints to be affected involve the knee, hip, hand, spine, neck and big toe. However, individuals with osteoarthritis may have one, a few or most of these sites affected. A recent report found the prevalence of symptomatic hand osteoarthritis in the general population to be 15.9% in women and 8.2% in men⁵.

GWAS and meta-analysis efforts have yielded several significantly associated loci for osteoarthritis of the hip and osteoarthritis of the knee^{6,7,8,9,10,11,12,13}. Thus far, no report has described genome-wide significant association with hand osteoarthritis, although the locus associated with knee osteoarthritis at 7q22 also confers risk of osteoarthritis of the hand⁶.

With the aim of discovering sequence variants that confer risk of osteoarthritis of the hand, we carried out a GWAS in subjects with severe hand osteoarthritis. We included 623 Icelanders with severe osteoarthritis of the hand (severe osteoarthritis at the thumb base and severe finger osteoarthritis in the same person) as cases and 69,153 individuals as population controls (see the [Methods](#) and [Supplementary Note](#) for a detailed description of phenotype and the sample set). We then tested for association between 34.2 million sequence variants, which were identified through whole-genome sequencing of 2,230 Icelanders and subsequently imputed into the cases and controls ([Methods](#)), and severe osteoarthritis of the hand. The most significant associations were with several common variants at 15q22 ([Supplementary Figure 1](#)), represented by rs12907038[G] (frequency of 50.7%, OR = 1.51, $P = 3.99 \times 10^{-10}$) and with very rare variants at 1p31 (NCBI Build36/hg18 chromosome 1, g.63807756[A], frequency of 0.022%, OR = 47.7, $P = 1.53 \times 10^{-9}$).

The group of variants at 15q22 included 55 variants with association $P < 5 \times 10^{-8}$, with the frequency of the risk alleles ranging between 41 and 54% ([Supplementary Table 1](#)). These variants are all located within a single linkage disequilibrium (LD) block that contains one gene, *ALDH1A2* (encoding aldehyde dehydrogenase 1 family, member A2) ([Figure 1](#)). We note that the top SNPs fell mainly within two frequency groups, around 41% and 52% for the risk allele, and were therefore not fully correlated with one another (for example, $r^2 = 0.62$ between rs4238326 (41%) and rs3204689 (52%)). Furthermore, the most strongly associated SNP, rs12907038, was triallelic and was likewise not fully correlated with the other two markers. Conditional analysis showed that markers in either frequency group alone could not account for the association signal; however, the triallelic rs12907038 SNP could fully account for the signal ([Methods](#) and [Supplementary Table 1](#)).

We tested SNPs from the two frequency groups, rs4238326 (41%) and rs3204689 (52%), in five additional European sample sets—the Genetics Osteoarthritis and Progression (GARP) study¹⁴, the Leiden Longevity Study (LLS)¹⁵ and Rotterdam I and II¹⁶ cohorts from The Netherlands and the TwinsUK¹⁷ and Chingford¹⁸ cohorts from the UK—as the triallelic marker rs12907038 was not present in these *in silico* replication data sets. Both markers

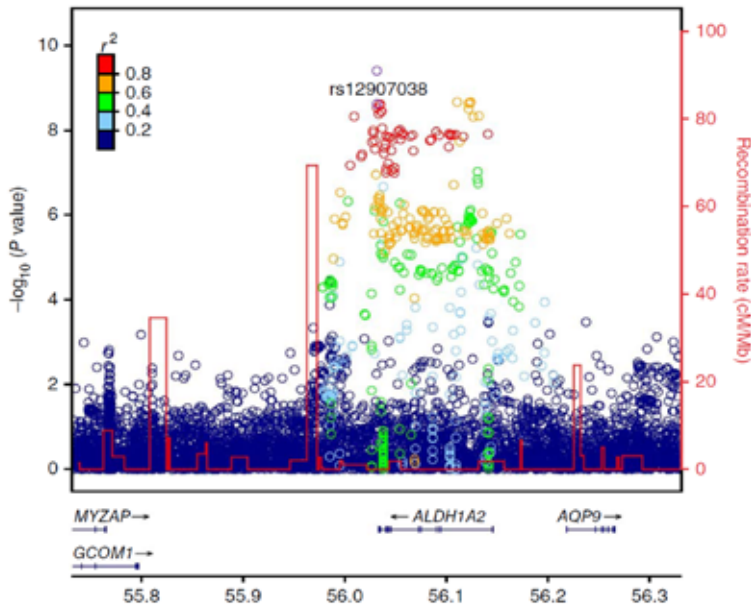


Figure 1. Regional association plot for the 15q22 ALDH1A2 locus.

P values ($-\log_{10}$) of SNP association with severe hand osteoarthritis in the Icelandic discovery samples are plotted against their positions at the 15q22 locus. The purple circle highlights the most significantly associated SNP, rs12907038, in the discovery analysis. SNPs are colored to reflect their LD with rs12907038 in the Icelandic data set. The red line indicates recombination rates, based on the Icelandic recombination map for males and females combined³⁵, with the peaks indicating recombination hotspots defining LD blocks in Icelanders. Known genes in the region are shown underneath the plot, taken from the UCSC genes track in the UCSC Genome Browser. All positions are in NCBI Build 36 coordinates. The plot was created using a stand-alone version of LocusZoom software³⁶.

were associated with severe hand osteoarthritis in the replication samples with $P = 0.0075$ for rs4238326 and $P = 0.0011$ for rs3204689 (Table 1 and Supplementary Table 2), yielding overall associations of $OR = 1.44$ and $P = 8.6 \times 10^{-11}$ for rs4238326 and $OR = 1.46$ and $P = 1.1 \times 10^{-11}$ for rs3204689 in the combined analysis of all samples (Table 1). In conditional analysis in the replication samples, rs3204689 was nominally significant after accounting for association at rs4238326 (Supplementary Table 3). We next examined association separately for severe finger osteoarthritis and for severe thumb osteoarthritis in all sample sets. The effects of the variants on severe finger osteoarthritis were comparable to those on severe thumb osteoarthritis ($OR = 1.26$ – 1.14), although, overall, the associations were somewhat more significant for the severe finger phenotype, with $P = 2.0 \times 10^{-11}$ and 5.6×10^{-12} compared to $P = 1.5 \times 10^{-8}$ and 5.1×10^{-7} for rs4238326 and rs3204689, respectively, for association with severe thumb osteoarthritis (Table 1). To determine whether this locus also associates with osteoarthritis of the larger joints (the hip and knee), we examined the results for these markers in large meta-analyses of hip osteoarthritis (4,349 cases and 46,903 controls) and knee osteoarthritis (5,224 cases and 48,571 controls) conducted by

Table 1. Markers at 15q22, rs4238326-C (freq. 41%) and rs3204689-C (freq52%), associate with severe hand osteoarthritis

Hand OA phenotype	marker	Discovery			Replication			Overall	
		OR	P value	N cases /controls	OR	P value	N cases /controls	OR (95% CI)	P value
Severe thumbs and severe fingers	rs4238326	1.48	2.3×10 ⁻⁹	623 / 69,153	1.33	0.0075	214 / 8,172	1.44 (1.29-1.60)	8.6×10 ⁻¹¹
	rs3204689	1.48	2.5×10 ⁻⁹		1.41	0.0011		1.46 (1.31-1.63)	1.1×10 ⁻¹¹
Severe Fingers	rs4238326	1.27	2.4×10 ⁻⁹	1,935 / 71,595	1.22	0.0017	713 / 8,095	1.25 (1.17-1.34)	2.0×10 ⁻¹¹
	rs3204689	1.26	7.7×10 ⁻⁹		1.26	0.000017		1.26 (1.18-1.34)	5.6×10 ⁻¹²
Severe Thumbs	rs4238326	1.23	1.8×10 ⁻⁶	1,610 / 74,060	1.19	0.0021	927 / 7,913	1.21 (1.14-1.30)	1.5×10 ⁻⁸
	rs3204689	1.21	8.4×10 ⁻⁶		1.14	0.014		1.18 (1.11-1.27)	5.1×10 ⁻⁷

The phenotypes of associations are shown, number of cases and controls, along with their respective P values and odds ratios (OR) and the 95% confidence interval (CI). The P values are corrected for genomic controls. The results are shown for the Icelandic discovery set, the replication sets combined (Rotterdam I and II, Garp, TwinsUK and Chingford) and the overall results for the discovery and replication combined.

the TreatOA (Translational Research in Europe Applied Technologies for Osteoarthritis) consortium¹³ (Methods and Supplementary Table 4). No association was observed with hip osteoarthritis (OR = 1.04, $P = 0.1$ for both markers). rs3204689 was nominally protective in the knee osteoarthritis data set (OR = 0.95, $P = 0.044$) (Supplementary Table 5). The lack of association with knee osteoarthritis is surprising in light of previous findings of correlations between hand osteoarthritis and knee osteoarthritis^{19, 20, 21}.

We explored the mRNA expression profile of the *ALDH1A2* gene in white blood cells, adipose tissue and articular cartilage collected during hip or knee joint replacement (Methods and Supplementary Note). The gene was not expressed in white blood cells and was expressed at low levels in adipose tissue, whereas it was highly expressed in cartilage samples. There was a correlation between the risk variant rs12907038[G] and decreased levels of the *ALDH1A2* transcript in adipose tissue ($P = 0.0017$, 4.1% decrease in expression) (Figure 2a). For rs4238326[C] and rs3204689[C], the reduction in expression of *ALDH1A2* in adipose tissue was similar at 3.7% per allele carried ($P = 0.0044$ and 0.0095 , respectively). Furthermore, we also tested whether there was an allelic imbalance in the expression of the gene in articular cartilage dependent on the presence of the risk allele. We used rs3204689 for this analysis, as it is located within the transcript (in the 3' UTR of the *ALDH1A2* transcript), and found that the transcript with the risk-conferring C allele was expressed at a lower level than the corresponding normal transcript. There was a 17.4% ($P = 0.00032$) and 11.6% ($P = 0.0016$) excess of transcript with the G allele compared to transcript with the risk-associated C allele in preserved (macroscopically normal) and osteoarthritic affected cartilage, respectively (Figure 2b and Supplementary Figure 2). These expression data support a role for *ALDH1A2* in mediating the effect of the osteoarthritis risk variants in the associated locus

The *ALDH1A2* gene encodes retinaldehyde dehydrogenase 2 (RALDH2), an enzyme that catalyzes the synthesis of retinoic acid from retinaldehyde (retinal). Retinoic acid, an active derivative of vitamin A (retinol), is a hormonal signaling molecule that has an essential role in embryonic development²² and in the maintenance of adult tissues²³, including cartilage and bone^{24, 25, 26}. The spectrum of activities for retinoic acid is broad and complex, controlling the expression of an array of target genes through binding of nuclear receptors (retinoic acid receptor (RAR) and retinoid X receptor (RXR)) or PPAR (peroxisome proliferator-activated receptor) β or δ , or through direct activation of kinase cascades^{27, 28}. Tight control of both spatial and temporal expression of all players in the retinoid metabolism pathway—and retinoic acid concentration in particular—is observed during development^{22, 29}, indicating a role for retinoic acid as a morphogen, which is supported by the observations that both excess and deficiency of retinoic acid at different time points can cause developmental anomalies^{22, 30}. Mice null for *ALDH1A2* (also known as *Raldh2*) display numerous abnormalities and die before midgestation. Limb bud patterning is one of the many abnormalities in these mice, presenting as hypoplastic or absent forelimb buds³¹. However, retinoic acid does not seem to be necessary for hindlimb development³². How retinoic acid could contribute to the osteoarthritic phenotype is poorly understood (Supplementary Note).

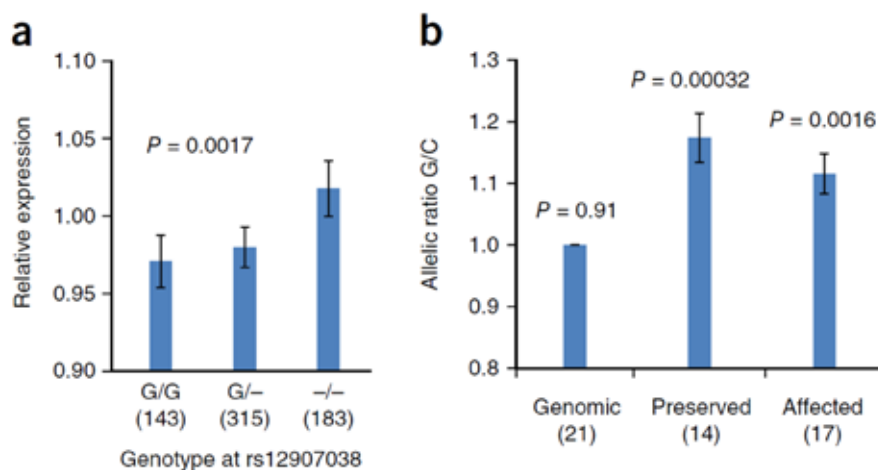


Figure 2. Genotype-dependent expression of *ALDH1A2* in adipose and cartilage tissues.

(A) The y axis shows average relative expression in adipose tissue, i.e., $10^{(\text{average MLR})}$, where MLR is the mean log expression ratio and the average corresponds to individuals with a particular genotype. The genotype allele “–” indicates the combined alleles A and C, and the number in parentheses indicates the number of individuals with a particular genotype. Error bars indicate s.e.m. P values were derived by regressing MLR values on the number of risk-conferring alleles of rs12907038[G] that an individual carries, adjusting for the effects of age and sex by including them as explanatory variables. (B) Allele-specific expression in genomic DNA and in preserved (macroscopically normal) and osteoarthritic affected cartilage tissue. The y axis shows the average ratio between the G and C alleles of rs3204689, and error bars indicate s.e.m. P values are derived from a one-sample t test of the mean differing from equal expression for transcripts carrying the G and C alleles (mean $\log_2$ (ratio) = 0).

Table 2. rs4238326 and rs3204689 are not associated with hip or knee osteoarthritis

Osteoarthritis phenotype	Marker	OR (95% CI)	<i>P</i> value	Cases	Controls
Hip osteoarthritis	rs4238326-C	1.04 (0.99-1.09)	0.10	4,349	46,903
	rs3204689-C	1.04 (0.99-1.09)	0.11		
Knee osteoarthritis	rs4238326-C	0.96 (0.91-1.01)	0.10	5,224	48,571
	rs3204689-C	0.95 (0.90-1.00)	0.044		

The results from meta-analyses of hip osteoarthritis and knee-osteoarthritis conducted by the TreatOA consortium. The P values are corrected for the overall meta-analyses genomic controls, $l = 1.028$ for hip and $l = 1.036$ for knee.

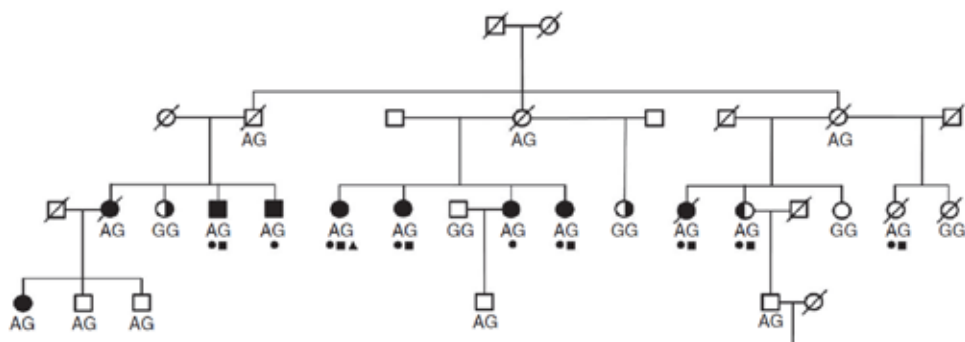


Figure 3. Pedigree of a family segregating the rare variants at 1p31.

AG and GG refer to the genotypes at chr. 1: 63,807,756: individuals with AG are carriers, and individuals with GG are non-carriers. Severe finger and thumb phenotype is represented by filled symbols; severe thumb and severe finger phenotypes are represented by vertically half-filled symbols, right and left, respectively. Osteoarthritis at other joints is shown below the symbols: box, knee; triangle, hip; dot, big toe. All individuals in generation III with available phenotype or genotype information are shown. The carriers in generations IV and V are between 38 and 65 years old and do not have clinical information available on hand osteoarthritis status, except for individual IV-I.

We improved imputation of the rare variants (0.02%) at the 1p31 locus that were associated with osteoarthritis by direct genotyping (Methods); this resulted in slightly stronger association with severe hand osteoarthritis ($OR = 50.6$ and $P = 9.8 \times 10^{-10}$ for chr. 1: 63,807,756) (Table 2) and with polyarthritis ($OR = 33.0$ and $P = 8.2 \times 10^{-8}$). Among the 53 carriers found in the Icelandic data set was a family with several members in whom extremely severe hand osteoarthritis segregated with the risk allele. These individuals also displayed osteoarthritis at several joints (Figure 3). All the affected carriers in the data set shared ancestors—a couple born in 1550—and all shared a 912-kb region at 1p31 that encompassed the three associated variants, seven genes and one pseudogene (Supplementary Figure 3). We could not find any exonic variant within these genes that the 3 associated intergenic variants tagged, even by carefully examining the whole-genome sequencing BAM files or resequencing the 13 exons that were not fully covered in the 2 carriers that underwent whole-genome sequencing. Neither did exome sequencing of nine carriers and nine non-carriers of chr. 1: 63,807,756 identify any tagging variant within this shared region. Of the three associated variants, chr. 1: 63,807,756 was predicted by ESPERR³³ to be within a functional element or an unknown exon. The SNP is within intron 12 of *EFCAB7* and is 46 and 53.7 kb upstream of *ITGB3BP* and *PGM1*, respectively (Supplementary Figure 3). Furthermore, intron 12 of *EFCAB7* is not spliced out in one transcript (ENST00000461039, Ensembl), and RNA sequencing also indicated that chr. 1: 63,807,756 lies within a potential exon in undifferentiated chondrocytes from the knee joint³⁴. Additionally, the variant overlapped MeDIP (methylated DNA immunoprecipitation)-seq methylated CpGs from a brain sample (Methods and Supplementary Note). In light of the resequencing results and

bioinformatics indication, this variant may be the functional variant that drives the association signal. However, this remains to be examined further. The chr. 1: 63,807,756 marker was present in samples from Sweden, the UK and The Netherlands at a slightly higher frequency than in samples from Iceland (0.1%). Hand osteoarthritis status was not known for all 15 carriers found in these samples, although 2 are known to have hand osteoarthritis and 4 have osteoarthritis of the knee. See the [Supplementary Note](#) for more data on the 1p31 locus.

We report here the results from our GWAS on severe hand osteoarthritis, using both common and rare sequence variants in the genome. Variants within the *ALDH1A2* gene associate significantly with severe osteoarthritis of the hand and yield the first genome-wide significant locus for hand osteoarthritis that replicates consistently, in five European sample sets. These variants confer relatively high ORs for common variants: 1.43–1.46. The transcript with the risk-conferring allelic variant of the gene is expressed at a lower level in cartilage and adipose tissue than its non-risk counterpart. We also describe a rare variant at 1p31 (0.02%, OR = 50) that segregates with severe hand osteoarthritis and generalized osteoarthritis in one Icelandic family with a large number of cases. The variant was also found in other populations where 6 of 15 carriers are known to have osteoarthritis of the hand or knee.

Methods

Study populations

We used 6 study populations for the *ALDH1A2* gene study: a discovery set of 69,786 Icelanders and 5 *in silico* replication sets, including 2 sample sets from the UK (the TwinsUK study¹⁷ of 1,557 women and the Chingford study¹⁸ of 578 women) and 3 Dutch samples (the GARP¹⁴ and LLS¹⁵ studies of 2,475 subjects, the Rotterdam I study¹⁶ of 3,137 persons and the Rotterdam II study¹⁶ of 1,184 persons). For the 1p31 locus, we used 9 study populations—the same discovery set of 69,786 Icelanders and 8 additional samples—to investigate whether the marker existed in other populations, some with limited information on severe hand osteoarthritis: the TwinsUK cohort ($n = 526$), the Dutch Rotterdam III cohort ($n = 3,068$), the GARP¹⁴ ($n = 490$), LLS¹⁵ ($n = 2,456$), Research Arthritis and Articular Cartilage (RAAK) study ($n = 150$) and PAPRIKA ($n = 971$) studies, and Swedish samples from 1,787 individuals (Malmö Diet and Cancer (MDC) study).

Hand osteoarthritis phenotype

The hand osteoarthritis cases included in the study were confined to individuals considered to have severe hand osteoarthritis. In the scoring of the fingers in the Icelandic discovery set, the main emphasis was placed on affected distal interphalangeal joints (DIP) 2 and 3 bilaterally, with proximal interphalangeal joints (PIP) 2 and 3 contributing to the assessment, and less emphasis was placed on other joints. Joint count, severity and

bilaterality contribute to severity. In the scoring of the thumb base, similar principles apply, but bilaterality was not a requisite for diagnosis. Previous surgery of the thumb base was automatically registered as severe hand osteoarthritis of the thumb base. Clinical severity scoring was adjusted for age, and slightly different comparison criteria thus had to be used according to the age at examination ([Supplementary Note](#)). The severe hand osteoarthritis cases in the replication sets had Kellgren Lawrence score ≥ 3 in at least one DIP bilateral and Kellgren Lawrence score ≥ 3 in at least one carpometacarpal joint of the thumb. Further description of the characteristics of the study populations is provided in the [Supplementary Note](#). All participants provided written informed consent, and we obtained approval from all institutional review boards to carry out the study.

Genotyping and association analysis of Icelandic samples

Genotyping and imputation methods and the association analysis method in the Icelandic samples were as described previously³⁸. In short, about 34.2 million sequence variants (30.6 million SNPs and 3.6 million indels) were identified through whole-genome sequencing of 2,230 Icelanders (to an average sequencing depth of $>10\times$) using the Illumina Genome Analyzer IIX and HiSeq 2000 instruments. These variants were then imputed into 95,085 Icelanders who had been genotyped with various Illumina SNP chips and phased using long-range phasing^{39,40}. With the use of Icelandic genealogy, probabilities of genotypes were furthermore predicted for 296,526 close relatives of the 95,085 chip-typed individuals using the nationwide Icelandic genealogy database. Cases and controls were derived from the chip-typed individuals and the probable genotypes of their relatives, matching controls to cases on the basis of how informative the imputed genotypes were. Association testing for case-control analysis was performed using logistic regression. To account for relatedness and stratification within the case and control sample sets, we applied the method of genomic control⁴¹. The inflation λ_g in the χ^2 statistic in each genome-wide analysis was estimated on the basis of a subset of about 300,000 common variants, and P values were adjusted by dividing the corresponding χ^2 values by this factor. For the traits reported here, the estimated inflation factors were 1.17 for severe fingers and severe thumbs, 1.36 for severe fingers and 1.23 for severe thumbs.

The threshold for genome-wide significance was set at a P value less than 1.5×10^{-9} ($= 0.05/34.2$ million variants), a conservative estimate as many of the variants tested were highly correlated.

Single-SNP genotyping of markers at 1p31 was carried out on the Centaurus (Nanogen) platform and by Sanger sequencing.

Genotyping and association analysis of replication samples

The replication sets were genotyped with the Illumina HumanHap300, HumanHap550 or HumanHap610 SNP chip, and the two replication SNPs at 15q22 were looked up in these data ([Supplementary Note](#)). Association testing for case-control analysis was performed using logistic regression. The marker chr. 1: 63,807,756 was genotyped by Centaurus assay in the MDC study and by TaqMan assay (Applied Biosystems) in the GARP, PAPRIKA,

LLS, RAAK and Rotterdam studies. Lookup was performed in 526 subjects in the TwinsUK cohort who had undergone whole-genome sequencing.

Meta-analysis

Results from multiple case-control groups were combined using a Mantel-Haenszel model¹³⁷ in which groups were allowed to have different population frequencies for alleles and genotypes but were assumed to have common relative risks (a fixed-effect model). Heterogeneity in the effect estimate was tested assuming that the estimated ORs for different groups followed a log-normal distribution and using a likelihood ratio χ^2 test with degrees of freedom equal to the number of groups compared minus one.

Conditional analysis at 15q22

When performing conditional analysis, testing a variant conditional on the expected genotypes of another variant, for the Icelandic data set, this analysis was carried out using the same software that was used in the GWAS analysis³⁸, including both the chip-typed individuals and those with genotype probabilities predicted on the basis of data from genotyped relatives. For the replication sample sets—Rotterdam I and II, the GARP study, and the TwinsUK and Chingford studies—the conditional analysis was performed using R software.

TreatOA meta-analysis

The hip osteoarthritis meta-analysis was as described in Evangelou *et al.*¹³, and the knee osteoarthritis meta-analysis was an extension to a previously published meta-analysis by the TreatOA consortium⁷. Definition of the hip osteoarthritis and knee osteoarthritis phenotypes⁴², the study samples and whole-genome genotyping platforms, quality control measures and imputation methods have been described previously^{6, 7, 10, 13, 43}. The studies included and their sample sizes are shown in [Supplementary Table 4](#). The hip osteoarthritis meta-analysis included 4,349 cases and 46,903 controls from 8 sample sets from 4 populations, and the knee osteoarthritis meta-analysis included 5,224 cases and 48,571 controls from 9 sample sets from 5 populations. All samples are of northern European descent. Genomic control was applied to each study before meta-analysis. Effect estimates for each study were synthesized using an additive model, and meta-analysis was performed using inverse variance fixed-effect models. *P* values reported for the hand osteoarthritis-associated SNPs at 15q22 have been corrected for the overall genomic controls for the meta-analyses: $\lambda = 1.028$ for hip osteoarthritis and $\lambda = 1.036$ for knee osteoarthritis. No between-study heterogeneity was observed for either SNP ($I^2 = 0$).

Expression analysis of the *ALDH1A2* gene in cartilage

Cartilage samples were derived from the ongoing RAAK study ([Supplementary Note](#)). In the current study, we used paired preserved and osteoarthritis-affected cartilage samples from donors undergoing joint replacement surgery for primary osteoarthritis in either knee or hip ([Supplementary Table 6](#) and [Supplementary Note](#)). DNA was isolated

using the Promega Wizard Genomic DNA Purification kit according to the manufacturer's protocol. RNA was collected using Qiagen mini columns according to the manufacturer's protocol. RNA was processed with the First-Strand cDNA Synthesis kit according to the manufacturer's protocol (Roche Applied Science). Quantitative RT-PCR (RT-qPCR) measurements were performed on the Roche LightCycler 480 II, using Fast-Start SYBR Green Master reaction mix according to the manufacturer's protocol (Roche Applied Science). An allele-specific real-time TaqMan assay (C__3232487_10, Life Technologies) was used to quantify the allelic ratio of *ALDH1A2* (rs3204689) in heterozygous samples. Allelic ratios were calculated using the formula^{44, 45}.

$$(2 - FAMC_t / 2 - VICC_t)$$

Ratios between the amounts of each allele in every sample were calculated for genomic DNA and cDNA. For each sample, the average allelic ratio for genomic DNA—which represents the 1:1 ratio—was used to normalize the cDNA ratio to generate a corrected allelic ratio, and the average over-repeated (up to seven) measurements for the same samples was used. To test whether there was a significant difference in the expression between alleles for a particular tissue, a one-sample *t* test was used to determine whether the allelic ratios, averaged over the cases, were different from the expectation of equal expression, i.e., $\log_2(\text{allelic ratio}) = 0$. Overall expression of *ALDH1A2* was determined by Illumina HT-12 V3 microarrays using standard methods. There were two probes, approximately 499 bp apart, on the array used for *ALDH1A2*. Levels of expression, as determined by mean normalized probe level values, for *ALDH1A2* probe 1 (mean level = 10.44, chromosome 9, position 58,245,807–58,245,857; hg19) and probe 2 (mean level = 8.51, chromosome 9, position 58,246,307–58,246,356; hg19), which were above the observed average expression levels of genes in the articular cartilage (the mean normalized probe level value of measured genes in cartilage was 7.4 with a range of 6.6–14.9) ([Supplementary Note](#)).

Expression analysis of the *ALDH1A2* gene in white blood cells and in adipose tissue

We investigated the expression of *ALDH1A2* in a data set that included RNA samples from the white blood cells of 1,002 Icelandic individuals and from adipose tissue of 673 individuals⁴⁶. Most of these individuals—973 with white blood cell samples and 646 with adipose tissue samples—had imputed genotypes for the 34.2 million variants identified in whole-genome sequencing. Correlation between expression and the genotypes of the variants was tested by regressing measured MLR (mean log expression ratio) values on the number of copies of the risk-associated allele an individual carried. Effects from age and sex were taken into account by including these variables as explanatory variables. For white blood cells, we also adjusted for differential blood cell count, as these variables correlated strongly with the expression of a large fraction of the genes measured⁴⁶. All *P* values were adjusted for the relatedness of the individuals by simulating genotypes through Icelandic genealogy as previously described⁴⁷. Resulting adjustment factors for the χ^2 statistic were 1.08 and 1.06 for adipose and whole blood, respectively. The gene was expressed below reliable detection limits in white blood cells. In adipose tissue, the gene was expressed at moderate levels, and rs12907038[G] was correlated with expression

levels, with each risk allele decreasing expression by about 4% ($P = 0.0017$). Although this correlation was not very strong, no other common variant located in or within 100 kb of *ALDH1A2* and not highly correlated with rs12907038[G] was more strongly correlated with its expression.

Reimputation of variants at the 1p31 locus

We validated imputation of the rare variants at the 1p31 locus that associated with severe hand osteoarthritis with $P < 1 \times 10^{-7}$ in the initial GWAS by directly genotyping likely and possible carriers and predicted non-carriers by Sanger sequencing or by Centaurus assays. Directly assessed genotypes were added to the genotypes for the 2,230 sequenced individuals, and the combined set was used as a training set for reimputation of the variants. This improved the imputation information scores for all four markers, and the association based on reimputed genotypes with severe hand osteoarthritis was slightly stronger for three of these variants, whereas one was no longer associated (Table 2).

Sanger sequencing of exons in the 1p31 shared region

Dye-terminator Sanger sequencing was performed with the Applied Biosystems Big-Dye Terminator v3.1 Cycle Sequencing kit with Agencourt AMPure XP and Agencourt CleanSeq for cleanup of the PCR and cycle sequencing products, respectively. AMPure XP and CleanSeq bead cleaning was performed on a Zymark Sciclone ALH-500 liquid-handling robot system. Tray dilutions for PCR setup and cycle sequencing setup were prepared on a Packard MultiprobeII HTEX liquid-handling robot system, and genomic DNA and PCR products were transferred into the respective trays using the Zymark Sciclone ALH-500. PCR and cycle sequencing reactions were performed on MJ Research PTC-225 thermal cyclers. For signal detection, Applied Biosystems 3730xl DNA Analyzers were used.

Exome sequencing of chr. 1: 63,807,756 carriers

DNA samples isolated from blood were prepared for exome sequencing using the Nextera Exome Enrichment method from Illumina. In short, 50 ng of genomic DNA was ‘tagmented’ using the Nextera transposome complex, generating adaptor-ligated DNA fragments ready for enrichment. All samples were labeled by barcode or index sequence as a part of the tagmentation process. Samples were quantified using PicoGreen measurements, and the quality of each sample was assessed using the Agilent Bioanalyzer. Up to 12 samples were pooled together in equal quantities before capture. A bait library of >340,000 95-mer probes was used to enrich a 62-Mb region of the genome containing the coding exons (>200,000 exons) of 20,794 genes, UTRs and some noncoding RNAs. Biotinylated probes were used for hybridization to the target sequences after capture using streptavidin beads. Captured DNA was further amplified using PCR, and the quality of the pooled, enriched sequencing libraries was measured by Bioanalyzer. Further quality assessment was performed by sequencing each 12-sample pool on an Illumina MiSeq instrument. This was carried out to optimize cluster densities for further sequencing and to assess the proportion of reads from each sample within the pool. Sequencing of pooled Nextera

libraries was performed on an Illumina HiSeq 2000 instrument. In general, each pool (12 samples) was sequenced on 4 lanes of the HiSeq instrument, generating approximately 150 Gb (10–15 Gb per sample) of raw data.

Bioinformatics analysis of 1p31

We carried out a search for overlaps between variant locations and known bioinformatics features. We retrieved data from the UCSC test browser (hg19, Build 37)⁴⁸ and the main UCSC browser (hg18, Build 36). We accessed feature tracks from tissues that might be relevant to hand osteoarthritis and from other tissues containing genome positional information and identified those features that overlapped with the SNP^{33, 49}.

URLs

LocusZoom, <http://csg.sph.umich.edu/locuszoom/>; R software, <http://www.r-project.org/>.

Accession codes

Expression data used in this study have been deposited in the Gene Expression Omnibus (GEO) database under accessions **GSE7965** and **GPL3991**.

Author contributions

The study was designed and results were interpreted by U.S., G.T., I.M., U.T. and K.S. Phenotype data and Icelandic subject recruitment was coordinated and managed by T.I. and H.J. D.H., S.M., T.S and A.V coordinated, managed, genotyped and analyzed the Twins UK and Chingford cohort samples. M.B., M.K., P.E.S. and I.M coordinated, managed, genotyped and analyzed the samples from the GARP, LLS, PAPRIKA and RAAK studies. B.H., F.R., A.U., S.B-Z. and J. v M. coordinated, managed, genotyped and analyzed the samples from the Rotterdam cohorts. S.L. coordinated and managed the samples from the MDC study. E.E. undertook the meta-analysis for the TreatOA consortium. G.M., U.T., G.T., and U.S. coordinated sequence data analysis, imputation and association analysis in Icelandic dataset. O.T.M. and S.A. performed exome sequencing and analysis. Shared haplotype analysis was performed by M.L.F. and A.K. Sanger sequencing of Icelandic samples and Centaurus genotyping of Icelandic and Swedish samples was carried out and analyzed by H.T.H and U.S. Expression experiments were carried out and analyzed by N.B., A.M.S., R.G.H.H.N., A.J., A.S., F.F.E. and M.T. The paper was drafted by U.S., G.T. and K.S. All authors contributed to the final version of the paper.

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Supplemental Data

<http://www.nature.com/ng/journal/v46/n5/full/ng.2957.html#supplementary-information>

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Chapter 3

Underlying molecular mechanisms of *DIO2* susceptibility in symptomatic osteoarthritis

Nils Bomer^{1,2, #}, Wouter den Hollander^{1, #}, Yolande F.M. Ramos^{1, #}, Steffan D. Bos^{1,3},
Ruud van der Breggen¹, Nico Lakenberg¹, Barry A. Pepers⁷, Annelies E. van Eeden¹, Arash Darvishan⁸,
Elmar W. Tobij^{1,2}, Bouke J. Duijnisveld⁴, Erik B. van den Akker^{1,5}, Bastiaan T. Heijmans^{1,3},
Willeke M.C. van Roon-Mom⁸, Fons J Verbeek⁹, Gerjo J.V.M van Osch^{6,7}, Rob G.H.H. Nelissen⁴,
P. Eline Slagboom^{1,2,3}, Ingrid Meulenbelt^{1,3}.

¹Dept. of Molecular Epidemiology, LUMC, Leiden, The Netherlands. ²IDEAL The

Netherlands. ³Genomics Initiative, sponsored by the NCHA, Leiden, The Netherlands.

⁴Dept. of Orthopaedics, LUMC, Leiden, The Netherlands. ⁵The Delft Bioinformatics Lab,

Delft University of Technology, Delft, The Netherlands. ⁶Dept. of Orthopaedics, Erasmus

MC, Rotterdam, The Netherlands. ⁷Dept. of Otorhinolaryngology, Erasmus MC, Rotterdam,

The Netherlands. ⁸Dept. of Human Genetics, LUMC, Leiden, The Netherlands. ⁹Dept. of

Imaging & BioInformatics, LIACS, Leiden, The Netherlands.

authors contributed equally

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Abstract

Objectives: To investigate how the genetic susceptibility gene *DIO2* confers risk to OA onset in humans and to explore whether counteracting the deleterious effect could contribute to novel therapeutic approaches.

Methods: Epigenetically regulated expression of *DIO2* was explored by assessing methylation of positional CpG-dinucleotides and the respective *DIO2* expression in OA affected and macroscopically preserved articular cartilage from end stage OA patients. In a human *in vitro* chondrogenesis model, we measured the effects when thyroid signaling during culturing was either enhanced (excess T3 or lentiviral induced *DIO2* over-expression) or decreased (Iopanoic Acid).

Results: OA-related changes in methylation at a specific CpG dinucleotide upstream of *DIO2* caused significant upregulation of its expression (Beta=4.96; $P=0.0016$). This effect was enhanced and appeared driven specifically by *DIO2* rs225014 risk-allele carriers (Beta=5.58, $P=0.0006$). During *in vitro* chondrogenesis *DIO2* overexpression resulted in a significant reduced capacity of chondrocytes to deposit extra cellular matrix (ECM) components, concurrent with significant induction of ECM degrading enzymes (*ADAMTS5*, *MMP13*) and markers of mineralization (*ALPL*, *COL1A1*). Given their concurrent and significant upregulation of expression, this process is likely mediated via HIF-2 α /RUNX2 signaling. In contrast, we showed that inhibiting deiodinases during *in vitro* chondrogenesis contributed to prolonged cartilage homeostasis as reflected by significant increased deposition of ECM components and attenuated upregulation of matrix degrading enzymes.

Conclusions: Our findings show how genetic variation at *DIO2* could confer risk to OA and raised the possibility that counteracting thyroid signaling may be a novel therapeutic approach.

Introduction

Osteoarthritis (OA) is a prevalent, complex, disabling disease of articular joints, characterized by degradation of articular cartilage and remodeling of the subchondral bone. There is no effective therapy to reverse or slow down the disease except for joint replacement surgery at the end stage. As a result, OA has a large detrimental impact on the quality of life of the elderly and causes a major burden on health and social care. [1] To allow development of new therapies, there is an on-going need for insight into the underlying mechanisms driving OA. Genetic studies provided evidence that genes orchestrating growth plate endochondral ossification play a underlying role in common OA susceptibility,[2] hence functional follow up approaches require focus on both the early developmental and late acting effects of these OA genes. A notable example is the deiodinase iodothyronine type 2 (D2) gene (*DIO2*) with the C-allele of the single nucleotide polymorphism (SNP) rs225014 (frequency ~ 0.35) located in the coding region that conferred consistent risk to OA.[3, 4] The gene product of *DIO2* is responsible for catalyzing the conversion of intracellular inactive thyroid hormone (T4) to active thyroid hormone (T3). T3 subsequently signals terminal maturation of growth plate chondrocytes leading to cell hypertrophy, cartilage matrix destruction mediated via upregulation of hypoxia inducible factor-2 α (*HIF-2 α*) and runt-related transcription factor-2 (*RUNX2*),[5, 6] mineralization of the cartilage and eventually formation of bone.[7] There are striking parallels between the chondrocyte signalling events that take place in the growth plate and those of hypertrophic chondrocytes in OA affected articular cartilage.[5] This has led to the hypothesis that with age and environmental stresses the propensity of the highly specialized, maturational arrested articular chondrocytes is affected by loss of epigenic control. Progression of age and disease could result in reactivation of genes involved in endochondral ossification, leading to loss and mineralization of articular cartilage, a process known to contribute to OA.[3, 8-10] Functional genomic studies showed high expression of *DIO2* mRNA and D2 protein levels in osteoarthritic as compared to healthy cartilage.[5, 11, 12] Furthermore, *DIO2* allelic imbalance was assessed and showed that the OA risk allele 'C' was more abundantly present in articular joint tissues than the wild-type allele 'T'. [11] In transgenic rats *DIO2* overexpression conferred risk to articular cartilage destruction.[13] The underlying mechanism how the *DIO2* SNP confers susceptibility to OA in humans remains, however, to be determined[14] but most likely acts via aberrant up-regulation of its expression. The *DIO2* locus in humans contains several putative CCCTC-binding factor (CTCF) binding sites, including one that is overlapping with the rs225014 location.[15] CTCF is considered to facilitate long-range chromatin interactions in order to insulate gene expression and distal transcriptional elements on the genome are brought in close proximity to transcriptional start sites (TSSs) of genes to inhibit expression.[16]

In the current study, we focus on regulatory mechanisms of *DIO2* expression in preserved and osteoarthritic human articular cartilage, thereby taking into account the *DIO2* risk allele. The direct effect of changes in *DIO2* expression on chondrocyte function and human cartilage extracellular matrix (ECM) homeostasis, is subsequently studied in human *in vitro* chondrogenesis models which should be considered a well defined system

for studying changes in the ECM when chondrocytes differentiate, become hypertrophic and start to exhibit cartilage debilitating expression patterns.

Materials and methods

The ongoing RAAK study is aimed at the biobanking of joint materials (cartilage, bone and where available ligaments) and mesenchymal stem cells and primary chondrocytes of patients in the Leiden University Medical Center. In the current study we used paired preserved and OA affected cartilage samples from 52 Caucasian end stage OA patients undergoing joint replacement surgery for primary OA (23 hips, 29 knees). For additional details on the RAAK study, cell cultures, RNA and DNA extraction, quantitative RT-PCR, ChIP and the data analysis, see the Supplemental Methods.

Electrophoretic Mobility Shift Assay

For Electrophoretic mobility shift assays (EMSAs) synthetic oligonucleotides containing the putative CTCF binding site were 5'-end labeled by γ -³²P-ATP and subsequently purified by gel filtration on Sephadex G-25 Medium columns. For additional details, see the Supplemental Methods.

Quantification of methylation

The methylated fraction of CpG dinucleotides was assessed with MALDI-TOF mass spectrometry (Epityper, Sequenom), a commonly applied method to quantify CpG methylation.[17-19] For additional details, see the Supplemental Methods.

Lentiviral constructs and transduction

C-terminal FLAG-tagged cys-D2 (kindly provided by Prof. Dr. Bianco [20]) was digested with *EcoR1* followed by Klenow-treatment and digestion with *Xba1*. Inserts were inserted into the *EcoRV-XbaI* sites of the pLV-CMV-IRES-eGFP Lentiviral backbone. For additional details, see the Supplemental Methods.

In vitro chondrogenesis

3D pellets were formed using 2.5×10^5 hBMSCs. Chondrogenesis was initiated in serum-free chondrogenic differentiation medium. From day 14 onwards cell pellets were maintained either in the standard chondrogenic differentiation medium or in the presence of T₃ (10 nM) or IOP (10 μ M). For additional details, see the Supplemental Methods.

Relative pixel intensity

The relative pixel intensity was computed by loading the photos into ImageJ (v.1.47).[21-23] For additional details, see Supplemental Methods.

Results

Epigenetic regulation of *DIO2* expression by CpG methylation in articular cartilage

Online available ChIP-seq data revealed multiple transcription factors to bind the rs225014 locus of which CTCF was predicted to bind with the highest certainty (Figure S1). To assess the regulatory properties of the CTCF binding site overlapping rs225014 and to test whether the rs225014 alleles directly affect the binding, we performed an electrophoretic mobility shift assay (EMSA). The putative CTCF sequence overlapping rs225014 (*DIO2*-CTCF1) was found not functional nor, for that matter, dependent of the rs225014 alleles (Figure S2, lane 1-6).

As no further TFs were confidently predicted to bind the rs225014 locus, we set out to elucidate putative regulatory mechanisms of *DIO2*, independent of the rs225014 base change. We, therefore, quantified expression of *DIO2* and methylation of 23 CpG dinucleotides across the *DIO2* locus in macroscopically preserved and OA affected cartilage from joints of patients undergoing total arthroplasty of the knee (N=29) or hip (N=23) (RAAK study; Table S1). We found borderline significant upregulation of *DIO2* expression (Beta=0.22, $P=0.063$, Figure 1C) in OA as compared to preserved cartilage. Furthermore, we observed several CpG dinucleotides to be differentially methylated between preserved and OA affected cartilage (Table S2). However, only for the CpG site 2031 base pairs upstream of the *DIO2* transcription start site (CpG -2031, Figure 1A), we observed significant differential methylation between OA and preserved cartilage (Beta=0.028, $P=0.0007$, Figure 1B) and a significant positive association between methylation and *DIO2* expression in all samples (Beta=4.959; $P=0.0016$, Figure 1D; Table S2). To confirm the regulatory properties of CpG -2031 on *DIO2* expression, we applied 5-aza-2'-deoxycytidine (AZA), a demethylating agent, to the culture medium in 8 primary chondrocyte cultures derived from preserved cartilage of total hip replacement patients. Addition resulted in a decrease in methylation at CpG -2031 ($P=0.003$, Figure 2A) corresponding to down regulation of *DIO2* expression ($P=0.004$, Figure 2B). Having observed the association between *DIO2* expression and OA associated methylation at CpG -2031, we applied a multivariate model to assess the individual effects of CpG -2031 methylation, joint site, sex, BMI, age and rs225014 alleles on *DIO2* expression in articular cartilage (Table S3). We could hereby ratify the association between increased CpG -2031 methylation and *DIO2* expression (Beta=4.008, $P=0.019$). The most notable observation, however, was the significant independent association of rs225014 genotype on *DIO2* expression (Beta=0.557; $P=0.0003$), indicating additive effects of both genotype and DNA methylation differences on *DIO2* expression. This effect appeared to be mainly driven by the risk allele. Upon stratification by the rs225014 alleles, no significant differences were observed between preserved and OA affected cartilage among homozygous carriers of the rs225014 wild-type allele T in methylation (Beta=0.018, $P=0.112$, Figure 1E) or expression (Beta=-0.017, $P=0.929$, Figure 1F). In carriers of the risk allele, however, an increase was observed in OA cartilage as compared to the preserved tissue, both in the difference of CpG -2031 methylation (Beta=0.034, $P=0.00002$, Figure 1H) and in expression (Beta=0.35, $P=0.012$, Figure 1I), concomitant with an increased association between methylation and ex-

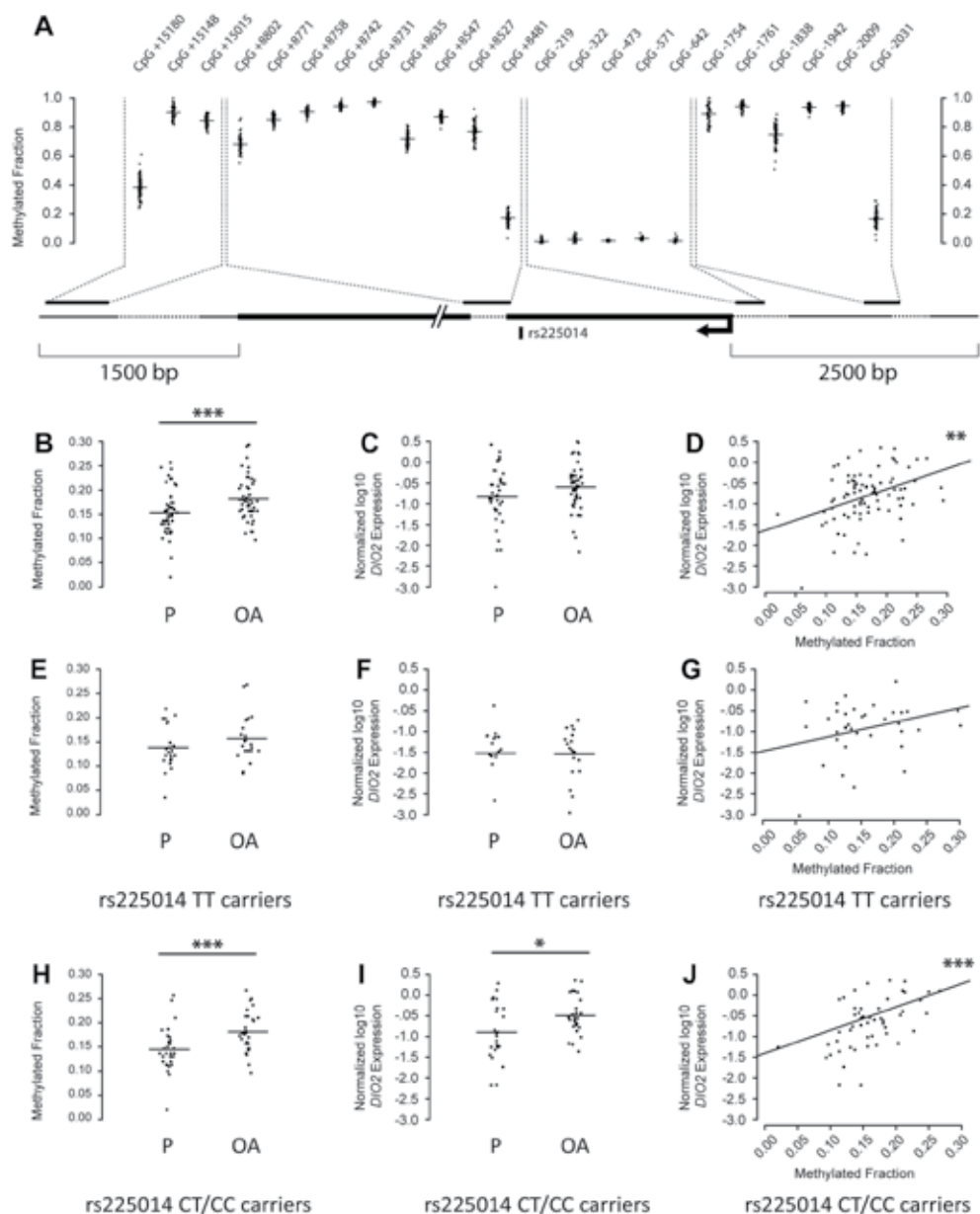


Figure 1. A functional CpG dinucleotide (CpG -2031) significantly modulates *DIO2* expression. (A) Schematic overview of the quantified CpG dinucleotides in pooled preserved and paired OA affected samples across the *DIO2* locus. (B) Methylation between preserved and OA affected samples for CpG dinucleotide located 2031 base pairs upstream (CpG -2031) of the *DIO2* TSS (GLMM, N=103, Beta=0.028, P=0.001, Bonferroni adjusted; see also Table S2). (C) Real-time qRT-PCR data of *DIO2* expression between preserved and OA affected cartilage (GLMM, N=87, Beta=0.22, P=0.063). (D) Association between methylation at CpG -2031 and *DIO2* expression (GLMM, N=87, Beta=4.959, P=0.001, Bonferroni adjusted; see also Table S2).

$P=0.002$ (Bonferroni adjusted for 23 CpG sites tested). (E) Methylation between preserved and OA affected cartilage for CpG -2031 among homozygous rs225014 wildtype allele carriers (GLMM, $N=44$, $\text{Beta}=0.018$, $P=0.112$). (F) Real-time qRT-PCR data of *DIO2* expression between preserved and OA affected cartilage among homozygous rs225014 wildtype allele carriers (GLMM, $N=36$, $\text{Beta}=-0.017$, $P=0.929$). (G) Association between methylation at CpG-2031 and *DIO2* expression among homozygous rs225014 wildtype allele carriers (GLMM, $N=36$, $\text{Beta}=3.863$, $P=0.050$). (H) Methylation between preserved and OA affected cartilage for CpG -2031 among the hetero- and homozygous carriers of the rs225014 risk allele (GLMM, $N=59$, $\text{Beta}=0.034$, $P = 0.00002$). (I) Real-time qRT-PCR data of *DIO2* expression between preserved and OA affected cartilage among rs225014 risk allele carriers (GLMM, $N=51$, $\text{Beta}=0.35$, $P=0.012$). (J) Association between methylation at CpG -2031 and *DIO2* expression among rs225014 risk allele carriers (GLMM, $N=51$, $\text{Beta}=5.58$, $P=0.0006$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). OA= osteoarthritic cartilage P= preserved cartilage.

pression ($\text{Beta}=6.816$, $P=0.00001$, Figure 1J). As expected, this association between expression and methylation was much smaller among homozygous wildtype allele carriers ($\text{Beta}=3.863$, $P=0.050$, Figure 1G).

***DIO2* effects on *in vitro* chondrogenesis; *DIO2* overexpression**

To assess the direct effect of *DIO2* upregulation upon cartilage matrix homeostasis, we examined *in vitro* chondrogenesis of human bone marrow derived stem cells (hBMSCs) in a pellet culture for 5 consecutive weeks and generated lentiviral-mediated overexpression of *DIO2* (Figure 3A and Figure S3). As a result, we observed a greatly reduced expression of genes encoding the main proteins of articular cartilage ECM, *COL2A1*, *ACAN* and *COL10A1*, by reverse transcriptase qPCR ($P<0.0001$; Figure 3A). In parallel, we observed profound and significant upregulated gene expression of the OA markers of hypertrophy and ECM breakdown *ADAMTS5*, *MMP13*, *RUNX2* and *EPAS1* (encoding *HIF-2 α*) from 3 weeks onwards ($P<0.05$, Figure 3A). Histology confirmed a reduced deposition of glycosaminoglycans (GAGs) (Figure 3B, 3C). Although we did not have data to apply proper statistics to produce a significant number, pixel intensity measurements of the pictures reproduced as in Figure 3 (B-C) showed us a 35% difference after 35 days between control and *DIO2* over-expressing pellets, concurrent with visual lower collagen type-II (*COL2*) and collagen type-X (*COL10*) protein expression by immunohistochemical staining at consecutive weeks as compared to controls (Figure 3D-G).

***DIO2* effects on *in vitro* chondrogenesis; thyroid hormone signaling**

To explore whether the effects of *DIO2* overexpression is due to enhanced thyroid signaling, we examined *in vitro* chondrogenesis of hBMSCs from 5 different donors (Table S4) in pellet cultures for 7 consecutive weeks (Figure S4) while adding active T3 to the chondrogenic medium from 2 weeks onwards (Figure 4A-F). Excess T3 resulted in a significant, progressive reduction in pellet sizes as compared to controls, at consecutive weeks of culturing ($P<0.05$, Figure 4G). Respective expression analyses showed a reduced expression of the ECM genes *ACAN* and *COL2A1* (Figure 4J), albeit that the downregulation of *COL2A1* appeared not statistically significant. Assessing the chondrogenic potential by measuring the ratio between *COL2A1* and *COL1A1* expression showed a dramatic effect

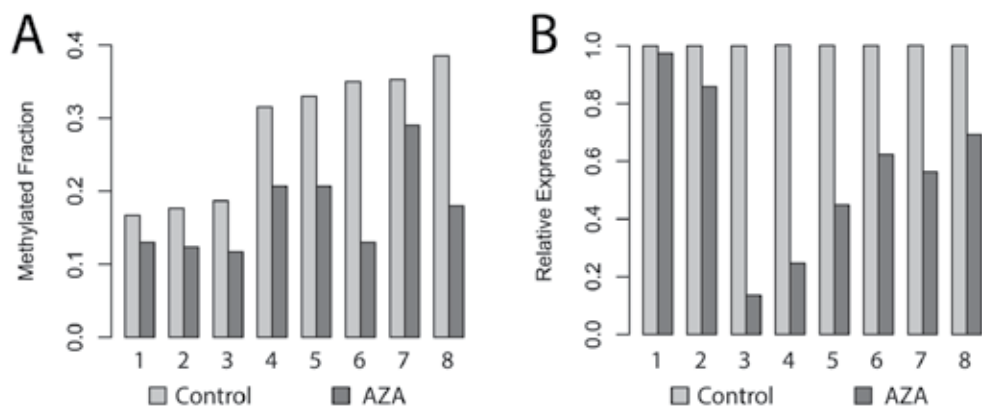
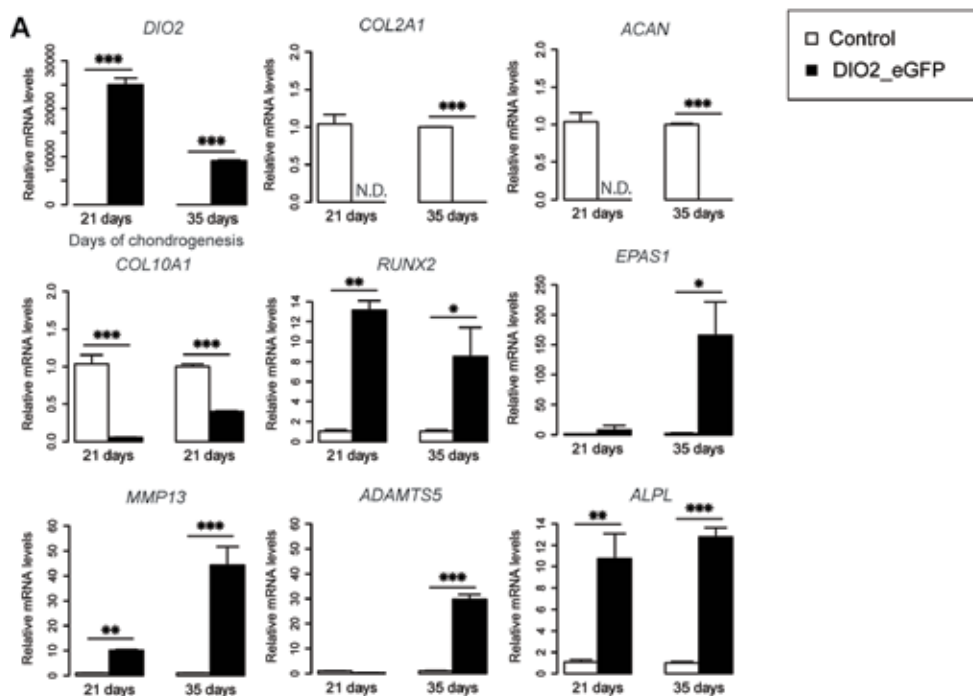


Figure 2. Methylation regulates *DIO2* expression in articular cartilage.

Each pair of bars reflects a unique donor cell culture, derived from total hip replacement patients.

(A) Methylation at dinucleotide CpG -2031 with no treatment and after treatment with 1.5 μ M of the demethylating agent 5-aza-2-deoxycytidine (AZA). (B) Real-time qRT-PCR data of *DIO2* expression with no treatment and after AZA treatment.



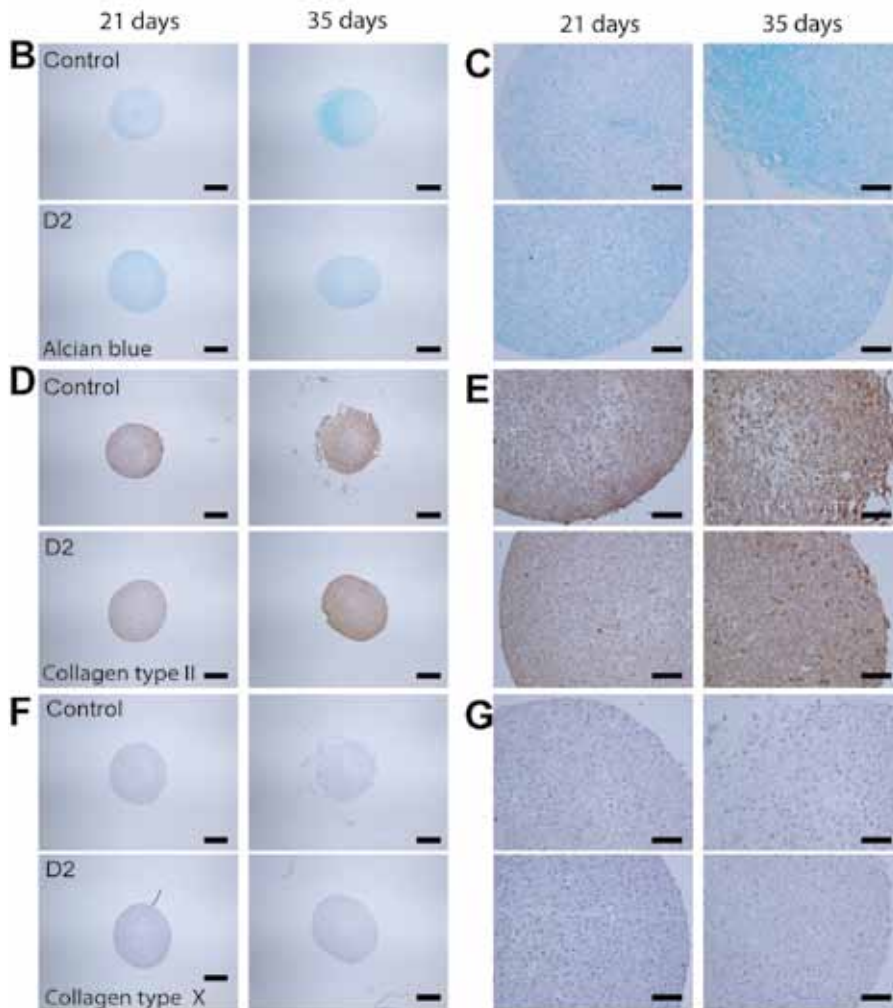


Figure 3. Overexpressing *DIO2* has a detrimental effect on cartilage extracellular matrix homeostasis.

(A) qRT-PCR analysis of *DIO2*, *COL2A1*, *ACAN*, *COL10A1*, *RUNX2*, *EPAS1*, *MMP13*, *ADAMTS5* and *ALPL* at 21 and 35 days of treatment in the chondrogenic hBMSC transduced with respectively control vector (eGFP) and *DIO2* (*DIO2_eGFP*; Figure S3). (B-G) Sections comparing control (top) and *DIO2* overexpressing (bottom) chondrogenic hBMSC pellets at 21 and 35 days of treatment.

(B-C) Alcian Blue staining. (D-E) Immunohistochemical staining of collagen type II. (F-G) Immunohistochemical staining of collagen type X. (B, D and F) Scale bar, 400 μm. (C, E and G) Scale bar, 100 μm. The expression levels were arbitrarily defined as '1' in the pellets grown under control conditions (white), and data from the pellets over-expressing *DIO2* (black) are given as the means $\pm$ s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. S.e.m. < 0.05 are not distinguishable in the figure.

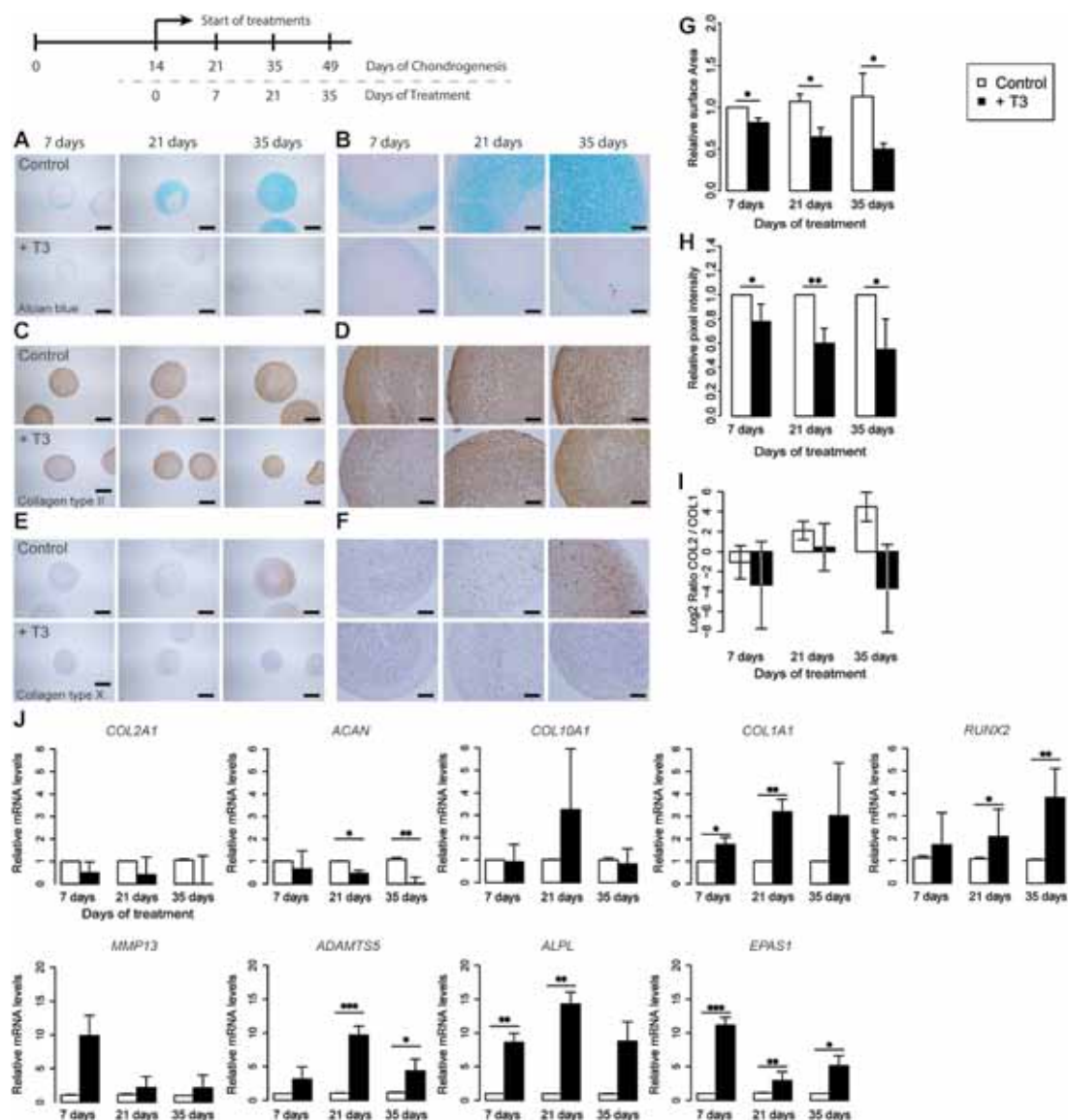


Figure 4. Thyroid hormone is believed to be a key regulator in human cartilage development.

(A-F) Sections of a representative donor (See Figure S4) comparing control (top) and T3 treated (bottom) chondrogenic hBMSC pellet at 7, 21 and 35 days of treatment. (A-B) Alcian Blue staining. (C-D) Immunohistochemical staining of Collagen type II. (E-F) Immunohistochemical staining of collagen type X. (A, C, and E) Scale bar, 400 μ m. (B, D and F) Scale bar, 100 μ m. (G) Mean surface area measurements at 7, 21 and 35 days of treatment with N = 3 donors in each group. (H) Mean quantitative pixel-intensity measurements (Figure S1) at 7, 21 and 35 days of treatment with N = 5 donors in each group. (I) Log2 Ratio of COL2A1 and COL1A1 with N = 5 donors in each group. (J) Mean real time qRT-PCR analysis of COL2A1, ACAN, COL10A1, COL1A1, ALPL, ADAMTS5, MMP13, RUNX2, and EPAS1 at 7, 21 and 35 days of treatment in chondrogenic hBMSCs of N = 5 donors in each group. The surface area measurement, pixel intensities and level of expression were arbitrarily defined as '1' in the pellets grown under control conditions (white) and the data from the pellets grown in the addition of excess T3 (black) are given as the mean $\pm$ s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001. S.e.m. < 0.05 are not distinguishable in the figure.

on the chondrogenic effect of the cell system upon addition of T3 (Figure 4I). Moreover, the OA markers of hypertrophy and ECM breakdown genes *ADAMTS5*, *RUNX2* and *EPAS1* were consistently up-regulated from 3 weeks of treatment onwards across the donor cultures ($P < 0.05$ Figure 4J). The differences in mean expression of *MMP13* between controls and T3 treated cells across the donors at consecutive timepoints was not significant. Immunohistochemical studies contributed to the confirmation that excess T3 resulted in a reduced expression of COL2 (Figure 4C-D) and COL10 (Figure 4E-F) and a pronounced reduction of GAGs (Figure 4A-B) as reflected by the reduced Alcian blue staining at consecutive time points with an overall mean decrease of 35% ($P = 0.0002$) as measured by the quantitative pixel-intensities (Figure 4H).

Pharmacological inhibition of D2

To investigate whether thyroid signalling blockade attenuates the detrimental effect on cartilage matrix homeostasis, pellet cultures were treated with the pharmacological deodinate inhibitor iopanoic acid (IOP) during *in vitro* chondrogenesis of hBMSCs of the 5 donors. Quantification of surface areas indicated increasing sizes at consecutive time points in the IOP-treated pellets comparable to non-treated, control pellets (Figure 5G). Respective expression analyses showed a significantly increased expression of the ECM genes *ACAN*, *COL2A1* and *COL10A1* after 3 weeks of treatment ($P < 0.01$, Figure 5J), whereas the OA markers of hypertrophy and ECM breakdown genes *ADAMTS5*, *RUNX2* and *EPAS1* were similar to control cultures (Figure 5J). Furthermore, we found no significant difference in chondrogenic potential between control and IOP treated pellets, when assessing the ratio between *COL2A1* and *COL1A1* expression (Figure 5I). Following these expression patterns, histological analysis of matrix components showed slightly higher levels of COL2 and COL10 staining (Figure 5C-F) and higher levels of GAGs as reflected by a significant 4% increase in Alcian blue staining at week 5 of treatment ($P = 0.018$; Figure 5A-B and Figure 5H). Of note is the denser cartilage matrix structure with less cellular lacunae at week 5 of treatment in cells treated with IOP as compared to controls (Figure 5A). By discriminating “cartilage” from the “lacunae” using the ImageJ data, we could show that administration of IOP resulted to an 10.92% decrease of lacunae ($P = 4.27 \times 10^{-5}$) on day 35 of treatment.

Discussion

In the current study, we provided insights into how genetic variation at the *DIO2* locus confers risk to OA. As a result of OA related changes in articular cartilage, loss of epigenetic silencing results in upregulation of *DIO2* expression among *DIO2* rs225014 risk-allele carriers (Figure 1, Figure 2). By applying an *in vitro* chondrogenesis model with genetically modified hBMSC, it was subsequently shown that genetic upregulation of *DIO2* expression resulted in a marked reduction of the capacity of chondrocytes to deposit ECM components, concurrent with induction of OA specific markers of cartilage matrix degeneration (*ADAMTS5* and *MMP13*) and mineralization (*ALPL*) (Figure 3). Given their concurrent upregulation, this process is likely mediated via *HIF-2 α* /*RUNX2* signalling, a hallmark of

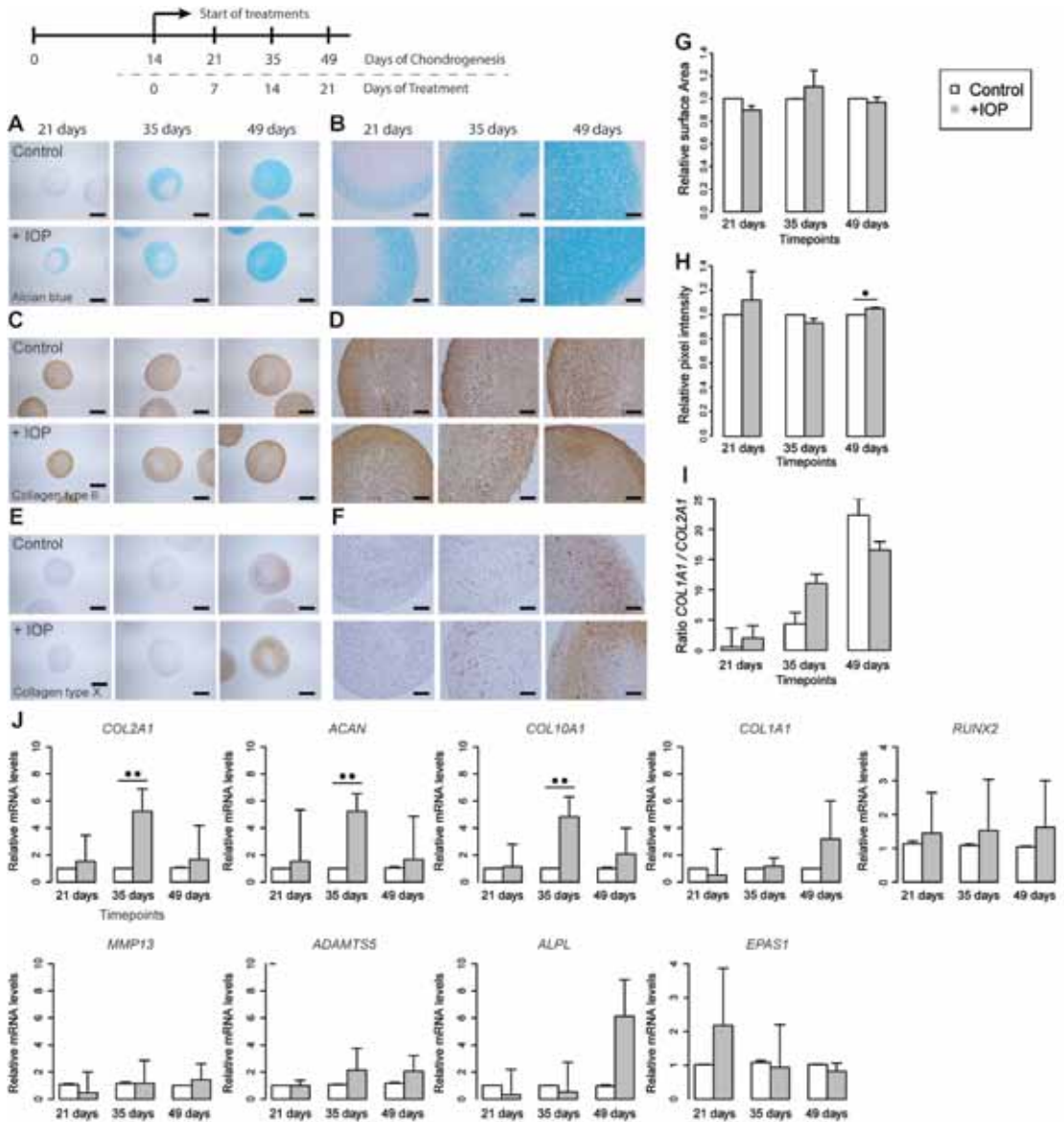


Figure 5. Counteracting thyroid signalling by inhibiting deiodinases in human cartilage development.

(A-F) Sections of a representative donor (See Figure S4) comparing control (top) and IOP treated (bottom) chondrogenic hMSC pellet at 7, 21 and 35 days of treatment. (A-B) Alcian Blue staining. (C-D) Immunohistochemical staining of Collagen type II. (E-F) Immunohistochemical staining of collagen type X. (A, C, and E) Scale bar, 400 μ m. (B, D and F) Scale bar, 100 μ m. (G) Mean surface area measurements at 7, 21 and 35 days of treatment with N = 3 donors in each group. (H) Mean quantitative pixel-intensity measurements (Figure S1) at 7, 21 and 35 days of treatment with N = 5 donors in each group. (I) Log2 Ratio of COL2A1 and COL1A1 with N = 5 donors in each group. (J) Mean real time qRT-PCR analysis of COL2A1, ACAN, COL10A1, COL1A1, ALPL, ADAMTS5, MMP13, RUNX2, and EPAS1 at 7, 21 and 35 days of treatment in chondrogenic hMSCs of N = 5 donors in each group. The surface area measurement, pixel intensities and level of expression were arbitrarily defined as '1' in the pellets grown under control conditions (white) and the data from the pellets grown in the addition of IOP (grey) are given as the mean $\pm$ s.e.m. * P < 0.05, ** P < 0.01, *** P < 0.001. S.e.m. < 0.05 are not distinguishable in the figure.

the OA disease process.[6, 24-26] Moreover, we show that the detrimental effects of *DIO2* upregulation are a result of increased T3 synthesis as reflected by the identical results when adding T3 to the culture medium (Figure 4). Given that the effects observed in both treatments are very similar and the fact that the specific downstream effect of D2 action is the conversion of inactive T4 to active T3, we are confident that the reported effects in both experiments are reflecting the same mechanism, albeit that we did not directly assess the levels of the trace-element T3 in our in vitro chondrogenesis model. Together our data are in line with our previous observations that carriers of the *DIO2* risk allele are prone to improper endochondral ossification and respective skeletal morphogenesis that could result in subtle malformations of joints or articular cartilage ECM composition.[27]

In contrast, we showed that inhibiting deiodinases by addition of IOP, contributed to prolonged 'healthy' cartilage homeostasis by virtue of attenuated upregulation of matrix degrading enzymes, a constant *COL2A1/COL1A1* ratio, denser cartilage matrix structure with significant less cellular lacunae which indicates a reduced propensity of chondrocytes to enter the terminal maturational process (Figure 5). In view of these findings, we advocate that attenuation of thyroid signalling by for example inhibiting deiodinases, could contribute to novel therapeutic options of OA or could improve outcomes of cartilage tissue-engineering approaches. Nevertheless, given that T3 has many and various biological functions, both in the circulation as well as in a tissue specific manner, local administration of an thyroid-blocking agent is likely necessary and a challenging aspect. Furthermore, the effect of, for example, IOP on other joint-tissues (e.g. ligament and synovium) requires investigation. It should be noted that IOP is a general inhibitor of deiodinases and as such could have also inhibited D1 and D3 action. In this respect, expression of *DIO1*, being important mainly in the circulation, is likely absent in cartilage tissues. Furthermore, by inhibiting D3, we prevented the conversion of active T3 to inactive T4 thereby ruling out the effect of T3 depletion due to inactive D2. Despite the beneficial effects of IOP, we observed upregulation of COL10 similar to control cultures reflecting the normal initiation of chondrocyte hypertrophy (Figure 5J). Vice versa, we showed that upon addition of T3, chondrocytes directly enter the terminal maturational process towards bone, as reflected by the up-regulation of enzymatic breakdown (*ADAMTS5*) and mineralization (*ALPL*, *COL1A1*), this without significant induction of COL10 deposition (Figure 4E-F). Together, these data indicate that chondrocyte hypertrophy in our model was not necessarily detrimental to cartilage homeostasis this in contrast to *DIO2* upregulation. Our data, therefore, indicates that upregulation of *DIO2* does not affect the 'early' hypertrophic expression of *COL10A1* but induces the later "progression stage"-marker *MMP13*, and the "late stage"-markers *ALPL* and *COL1A1*.[28] In our model, we used the expression of *EPAS1* and *RUNX2* to shown downstream effects of up-regulation of *DIO2* and observed a significant positive association between T3 and *EPAS1*. Although such an observation based on association does not imply a direct causal relationship, recent data of Chatonnet et al., (2013)[29] showed, in a ChIP-seq analysis in mouse C17.2 neural progenitor cells, that *EPAS1*, harbours specific thyroid hormone nuclear receptor (THR) binding sites and is directly reactive to thyroid hormone. In view of these data, we advocate that active thyroid hormone, likely by local *DIO2* action, could have an important

impact on *EPAS1* up-regulation during the pathophysiology of OA. Additional studies are, however, necessary to elucidate whether T3 is directly affecting expression of *EPAS1*, or *RUNX2* for that matter, by binding to a positional thyroid receptor in humans.

Despite the fact that the direction of gene expression changes of *COL2A1* and *MMP13* upon addition of T3 to the culture media appeared consistent with our overall results (Figure 4J), the mean differences in expression were not statistically significant across the donors. Most likely this was the result of the considerable heterogeneity in the differential gene expression patterns of these specific genes across donors, especially with respect to timing the respective down and up-regulation at consecutive time-points (Figure S4), a phenomenon generally recognized in the *in vitro* chondrogenesis models of primary hBM-SCs[30].

We detected several CpG dinucleotides across the *DIO2* locus that were differentially methylated between preserved and OA affected cartilage. Although we have only verified their functionality with respect to *DIO2* expression, we cannot exclude that methylation at these sites regulates expression of more distal genes. We found a consistent positive correlation between methylation at CpG -2031 and *DIO2* expression in articular cartilage among carriers of the rs225014 risk allele, which may not comply to the conventional inverse relation between CpG methylation and gene expression. However, in recent genome wide approaches, it has been recognized that this conventional relation primarily holds among CpG dinucleotides residing in CpG islands and proximal promoters, whereas gene body and distal enhancer methylation, as is the case for CpG -2031, has been shown to correlate in either direction with gene expression. [31-33]

With respect to our observations of *DIO2* expression in preserved and osteoarthritic cartilage, it should be noted that, in contrast to previously reported high upregulation of *DIO2* expression in osteoarthritic when compared to healthy cartilage[5, 11] we showed a moderate upregulation in osteoarthritic when compared to preserved cartilage of the same joint only among carriers of the rs225014 risk allele (Figure 1I). This difference suggests that upregulation of *DIO2* expression may be an early event in OA pathophysiology and might be set to continue progressively among rs225014 risk allele carriers.

We were unable to validate the functionality of a putative CTCF binding site directly at the rs225014 locus nor for that matter the causality of the previously assessed consistent allelic imbalance.[11] Possibly, the rs225014 SNP affects three dimensional chromatin conformations underlying the relation between the rs225014 tagged allelic imbalance and methylation dependent upregulation of *DIO2* among rs225014 risk allele carriers.

In conclusion, our data provides evidence in humans that genetic predisposition combined with early OA related changes results in loss of epigenetic silencing of *DIO2*, which likely induces *EPAS1* and *RUNX2* mediated up-regulation of cartilage matrix degrading enzymes (*ADAMTS5* and *MMP13*) and mineralization of matrix (*ALPL* and *COL1A1*), thereby driving the OA process most distinctly among *DIO2* risk allele carriers. Furthermore, our data shows that counteracting the thyroid signalling by inhibiting deiodinases could contribute to needed novel therapeutic approaches of OA.

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Competing interest

The authors declare no competing financial, personal, or professional interests.

Author contributions

Conceived and designed the experiments: NB; WdH; YFMR; FJV; PES; IM

Performed the experiments: NB; WdH; YFMR; RvdB; NL; AEvE; AD

Analyzed the data: NB; WdH; YFMR; AEvE; AD

Contributed reagents/materials/analysis tools: SDB; BAP; BJD; RGHHN

Wrote the manuscript: NB; WdH; YFMR; IM

Critically reviewed the manuscript: All authors

Supplemental Data

<http://ard.bmj.com/content/74/8/1571/suppl/DC1>

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Chapter 4

The effect of forced exercise on knee-joints in *Dio2*^{-/-}-mice; type II iodothyronine deiodinase-deficient mice are less prone to develop OA-like cartilage damage upon excessive mechanical stress

Nils Bomer^{1,2#}, Frederique M.F. Cornelis^{4#}, Yolande F.M. Ramos¹, Wouter den Hollander¹, Lies Storms⁴, Ruud van der Breggen¹, Nico Lakenberg¹, P. Eline Slagboom^{1,2,3}, Ingrid Meulenbelt^{1,3‡}, Rik J.L. Lories^{4,5‡}

¹Dept. of Molecular Epidemiology, LUMC, Leiden, Netherlands; ²Integrated research of Developmental determinants of Ageing and Longevity (IDEAL), Leiden, Netherlands; ³The Netherlands Genomics Initiative, sponsored by the NCHA, Leiden-Rotterdam, The Netherlands; ⁴Laboratory of Tissue Homeostasis and Disease, Skeletal Biology and Engineering Research Centre, KU Leuven, Leuven, Belgium. ⁵Division of Rheumatology, University Hospitals Leuven, Leuven, Belgium

#Shared first author; ‡Shared last author

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Abstract

Objective: To further explore deiodinase iodothyronine type-2 (*DIO2*) as a therapeutic target in osteoarthritis by studying the effects of forced mechanical loading on *in vivo* joint cartilage tissue homeostasis and the modulating effect herein of *Dio2* deficiency.

Methods: Wild-type and C57BL/6-*Dio2*^{-/-}-mice were subjected to a forced running regime for one hour per day for three weeks. Severity of osteoarthritis was assessed by histological scoring for cartilage damage and synovitis. Genome wide gene expression was determined in knee-cartilage by microarray analysis (Illumina MouseWG-6 v2). STRING-db analyses were applied to determine enrichment for specific pathways and to visualize protein-protein interactions.

Results: In total, 158 probes representing 147 unique genes showed significantly differential expression with a fold-change ≥ 1.5 upon forced exercise. Among these are genes known for their association with OA (e.g. *Mef2c*, *Egfr*, *Ctgf*, *Prg4* and *Ctnnb1*), supporting the use of forced running as an OA-model in mice. *Dio2*-deficient mice showed significantly less cartilage damage and signs of synovitis. Gene expression response upon exercise between wild-type and knockout-mice was significantly different for 29 genes.

Conclusion: Mice subjected to a running regime have significant increased cartilage damage and synovitis scores. Lack of *Dio2* protected against cartilage damage in this model and was reflected in a specific gene expression profile, and either mark a favorable effect in the *Dio2*-knockout (e.g. *Gnas*) or an unfavorable effect in wild-type cartilage homeostasis (e.g. *Hmbg2* and *Calr*). These data further support *DIO2* activity as a therapeutic target in OA.

Introduction

Osteoarthritis (OA) is a prevalent, complex, chronic and disabling disease of articular joints, characterized by progressive destruction of joint cartilage, remodeling of the subchondral bone, formation of osteophytes and synovitis [1, 2]. It causes pain and disability to an increasing proportion of the population and is associated with the obesity pandemic, aging of the population and not in the least by improved survival of patients with cardiovascular or oncological health problems. All together this imposes a large and growing social and economic burden [3, 4]. The development of novel therapeutic approaches is therefore urgently needed and should be based on insights into the underlying disease mechanisms [5-7]. Several genetic studies identified robust signals for OA susceptibility [8-13], that suggest specific genes, involved in cartilage development and growth, to play a key role in the OA disease process [14, 15]. Notable example of such OA susceptibility alleles, are the C-variant of the rs225014 single nucleotide polymorphism (SNP) located in the coding region of the deiodinase iodothyronine type-2 (D2) gene (*DIO2*) [8, 16] and the rs945006 SNP in the deiodinase iodothyronine type-3 (D3) gene (*DIO3*), another deiodinase with a counter regulatory function for *DIO2* [16]. For together, D2 and D3 primarily regulate the bio-availability of intracellular thyroid hormone in specific tissues such as the growth plate, but not systemically. The deiodinase type 2 protein (D2) catalyzes the conversion of intracellular inactive thyroxine (T4) to active thyroid hormone (T3). During skeletal development, this conversion plays a critical role in the process of endochondral bone formation by facilitating terminal maturation of hypertrophic chondrocytes subsequently leading to breakdown of the cartilage matrix and replacement by bone. This process is essential in skeletal development and growth but loss of the chondrocyte's maturational arrested characteristics is considered deleterious for postnatal articular cartilage.[17, 18] *DIO2* mRNA and D2 protein levels are highly upregulated in human osteoarthritic cartilage as compared to healthy cartilage [19-21], suggesting that in disease *DIO2* contributes to the loss of the highly specialized maturational arrested state of articular chondrocytes [17]. Cartilage-specific over-expression of human *DIO2* in rats was associated with increased damage to the articular cartilage in a surgical OA model. However, this was without clear evidence that hypertrophy of chondrocytes plays an essential role and rather pointing towards increased tissue destructive enzyme activity and enhanced expression of IL-1 target genes [22]. Upregulation of *DIO2* expression in a human *in vitro* model resulted in a marked reduction of the capacity of chondrocytes to deposit ECM components, including type II and type X collagen, while inducing OA-specific markers of cartilage matrix degeneration and mineralization [18]. In contrast, pharmacological inhibition of *DIO2* increased the expression of collagens and aggrecan without clear effect on hypertrophy or tissue destructive enzymes. These accumulating data suggest that D2 inhibition and/or modulation may become a therapeutic target, but the *in vivo* impact of D2 loss of function in joint biology and disease remains largely unknown in particular at the molecular level.

In this study, we set out to study the molecular network of *Dio2* in the healthy and challenged joint. We performed genome wide expression analyses in aging wild-type

and *Dio2*^{-/-} mice, including groups exposed to a moderately strenuous running regime. Our results indicate that *Dio2* is effectively involved in specific gene networks that can be associated with osteoarthritis and provide further insights into the complex molecular interactions involved in healthy and diseases articular cartilage.

Materials and methods

Animal experiments

Dio2^{-/-} mice were a kind gift of Dr. V. Galton (Dartmouth Medical School, NH, USA)[23] and were backcrossed onto the C57Bl/6 background. All experiments were approved by the Ethics Committee for Animal Research (KU Leuven, Belgium).

Four to 6 months old male *Dio2*^{-/-} (n=22) and wild-type mice (n=30) ran for 3 weeks 1 hour/day, 5 days/week, at a speed of 11 m/min and with an inclination of 5°. For additional details, see the Online Methods.

Histological assessment of osteoarthritis

Right knees were fixed overnight at 4°C in 2% formaldehyde, decalcified for 3 weeks in 0.5M EDTA pH 7.5 and embedded in paraffin. Severity of disease was determined by histological scores on hematoxylin/eosin or Safranin O stained sections (5µm) throughout the knee (5 sections at 100µm distance). For additional details on histological assessment and statistical analyses, see the Online Methods.

RNA isolation

Snap frozen cartilage of the left knees was powderized using a Retsch Mixer Mill 200 under cryogenic conditions. RNA was isolated and washed using the RNeasy mini kit (Qia-gen, Venlo, The Netherlands) according to the manufacturer's protocol. RNA quality was assessed using a Biocore lab-on-a-chip and quantity was assessed using a Nanodrop spectrophotometer. For additional details, see the Online Methods.

Microarray analysis

Complementary DNA synthesis, amplification, biotin labeling and hybridization onto the microarrays was performed using the Ambion TotalPrep-96 RNA amplification kit (Life Technologies, Bleiswijk, The Netherlands) according to manufacturer's protocol. After hybridization on Illumina MouseWG-6 v2 BeadChip microarrays (Illumina, Eindhoven, The Netherlands) the slides were scanned with the Illumina Beadscanner 500GX. For additional details on microarray handling and data analyses, see the Online Methods.

Pathway analysis and protein-protein interaction networks

Gene enrichment among the genes with significant differential expression was performed with STRING (Search Tool for the Retrieval of Interacting Genes/Proteins 9.1) [24]. Pathways with a P-value ≤ 0.05 after FDR correction were considered significant. Enrichment in protein-protein interactions was also analyzed using the STRING database. For addi-

tional details, see the Online Methods

Quantitative RT-PCR assays (validation)

Validation of the microarray results was performed by quantitative real-time PCR. 500 ng of total RNA was processed with the First Strand cDNA Synthesis Kit according to the manufacturer's protocol (Roche Applied Science, Almere, The Netherlands) upon which cDNA was diluted 5 times. RT-qPCR measurements were performed on the Roche Lightcycler 480 II, using Fast Start Sybr Green Master reaction mix according to the manufacturer's protocol (Roche Applied Science). For additional details, see the Online Methods

Results

Reduced severity of OA after forced running in *Dio2*^{-/-} mice

To study the role of *Dio2* in joint homeostasis, *Dio2*^{-/-} and wild-type mice were studied in a forced-exercise setup and compared to non-running mice (Figure 1). No striking developmental skeletal phenotype appears present in the *Dio2*^{-/-} mice [25] and our observations in our mouse colony are in agreement with these findings. Wild-type and *Dio2*^{-/-} mice displayed a similar running behavior. As shown in Figure 1, overall group analysis indicated a significant difference between wild-type- and knockout-mice with respect to cartilage damage ($P = 0.0006$) and synovial hyperplasia ($P = 0.0536$). No specific effects within wild-type- or knockout-mice of exercise were found, nor interaction between genotype and exercise. Sidak's multiple comparison test indicated that the effect was determined by the difference in genotype in the exercise group ($P < 0.0001$ for cartilage damage – 95% CI of the difference between the means (0.1820 to 0.5820) and $P = 0.0184$ for synovial hyperplasia – 95% CI of the difference between the means (0.03661 to 0.4627)) (Figure 1).

Microarrays: Differential expression in knee-joints upon forced exercise

To identify genes responsive to the forced running regime in mice, genome wide gene expression in knee-cartilage was studied by microarray. Gene expression was detected before and after the running regime in interaction with the genetic background (wild-type and *Dio2*^{-/-}-mice). Figure 2 shows a schematic overview of the study strategy.

After quality control and normalization, 20872 of the 45281 probes of the BeadChip array were used for analyses. Microarrays on human articular cartilage samples showed corresponding numbers for specific expression [26]. In order to detect all articular cartilage genes that are responsive to the applied running regime, we performed differential expression analyses in 3 strata: total (A), *Dio2*^{-/-} (B) and wild type (C) group (Figure 2). For each strata significance was adjusted for multiple testing according to the “Benjamini and Hochberg” method. In the total and wild type strata, we independently detected respectively 1862 and 892 probes representing 1699 and 830 genes (558 overlapping) that were significantly differentially expressed between the forced exercise and the control group. In contrast, in the *Dio2*^{-/-}-stratum, this comparison did not result in any significantly differentially expressed probes after multiple testing. Among the differentially ex-

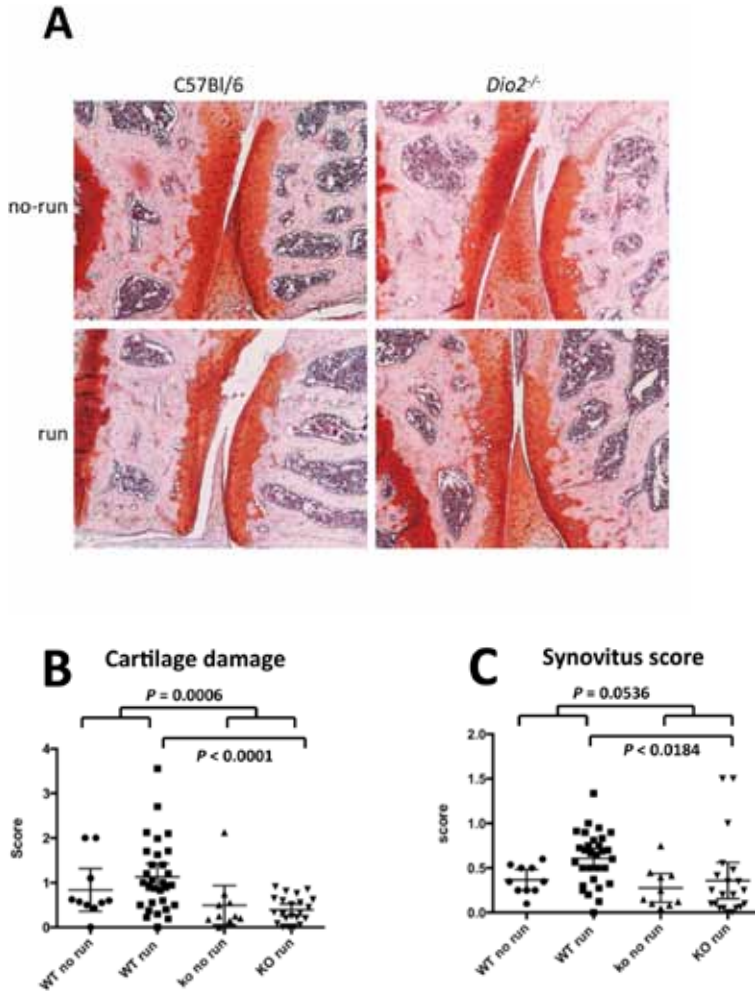


Figure 1. Histological scoring of osteoarthritis.

Comparing wild-type and *Dio2^{-/-}* mice as well as the effect of forced running. (A) Frontal Haematoxylin-Safranin O stained sections of C57Bl/6 wild-type and *Dio2^{-/-}* knees (medial) of mice subjected to a running regime (run) and control mice (no run) (magnification 10x). (B) Cartilage damage was increased in wild-type mice as compared to *Dio2^{-/-}* mice (2-way ANOVA $p = 0.0006$) and (C) a similar trend was observed for synovitis (2-way ANOVA $p = 0.0536$). None of the other comparisons (e.g. 'WT no run' versus 'KO no run') were found significantly different. Data are shown as individual values, mean and 95% confidence intervals.

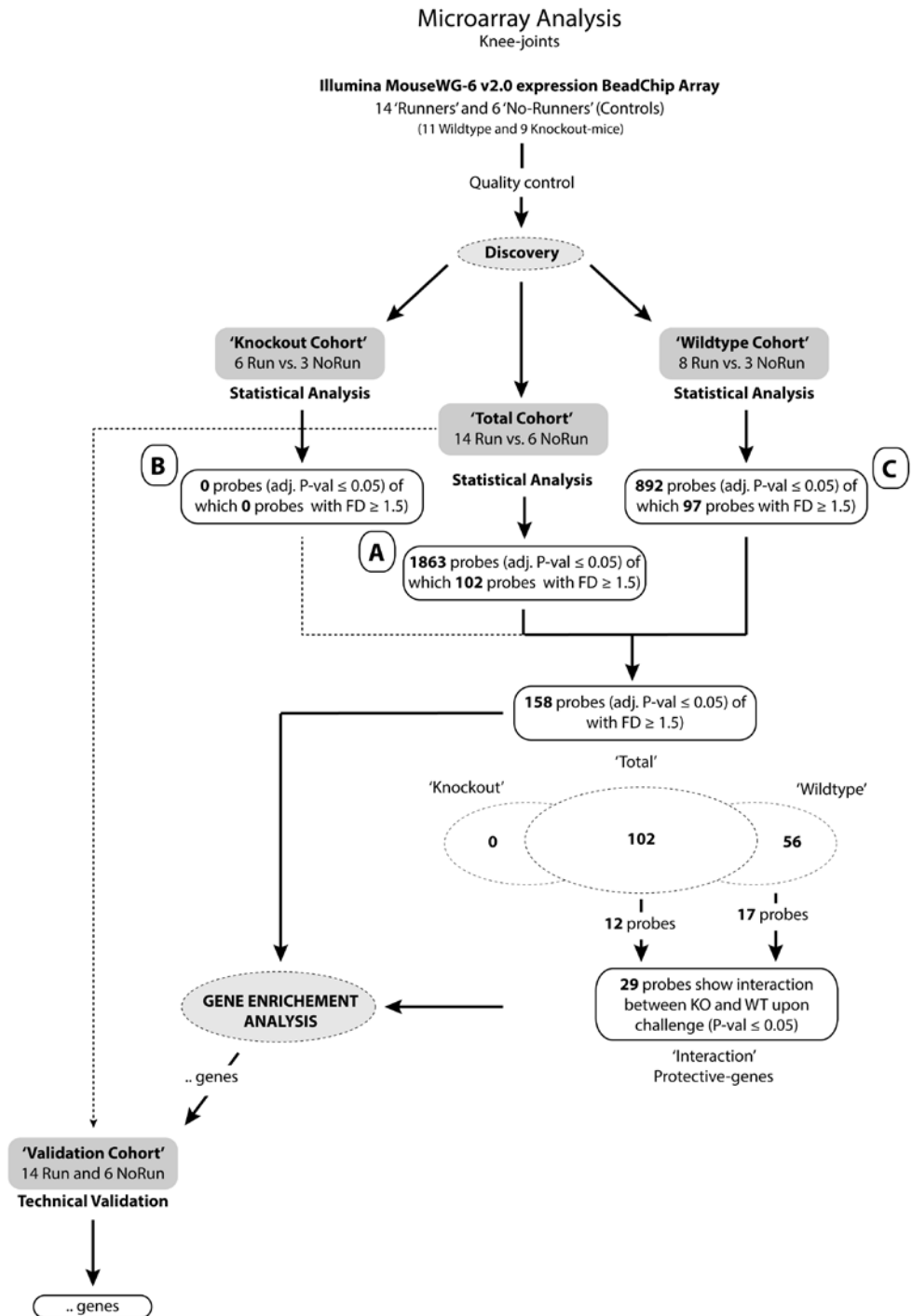


Figure 2. Overview of the study strategy.

Following microarray analysis, data were statistically analyzed. Secondary analyses using gene enrichment analysis and interaction analysis will lead to a set of genes which will be technically validated.

pressed probes in the total and wild-type groups, we observed respectively 102 and 97 probes with a fold-change of 1.5 and higher that together consisted of 158 significant unique probes, representing 147 unique transcripts that were responsive to the running regime (Figure 2 and Supplement Table S1). Notably, of the 158 differentially expressed probes, 31 were up-regulated (20%) and 127 showed down-regulation (80%). Among the 147 unique transcripts, we observed genes known for a potentially role in cartilage homeostasis and disease, such as Proteoglycan 4, also known as Lubricin (*Prg4*, 1.95-fold down; $P=4.44 \times 10^{-2}$)[27], myocyte enhancer factor 2C (*Mef2c*, 1.9-fold down; $P=3.05 \times 10^{-2}$)[28, 29] and connective tissue growth factor (*Ctgf*, also known as *Ccn2*, 1.79-fold down; $P=1.32 \times 10^{-2}$)[30].

Technical validation of our microarray results was carried out by RT-qPCR in the discovery cohort (20 samples previously included in the microarray analyses). Genes to be validated were selected based on P-value and fold difference (differential reaction on mechanical stress). Here, 19 out of the 20 genes tested showed similar effect sizes and direction as the original data, only *Pfn1* did not show similar effect of expression (see Supplementary Table S2).

Pathway analyses: Protein-protein interactions and gene enrichment analyses

To identify functional connections between the genes that were found as differentially expressed (Table S1) we determined the protein-protein interaction prediction using STRING-db. Analyzing the 147 differentially expressed genes, representing 124 unique proteins, showed enrichment of interaction in the subset of proteins ($P=1.61 \times 10^{-4}$; 63 interactions; Figure S1), indicating that the gene-products that were identified are closely interacting as a response to the forced running regime.

Furthermore, we assessed whether the differentially expressed genes occurred more frequently in a specific pathways in mice. Gene enrichment analyses revealed enrichment for biological processes (GOTERM_BP_FAT) concerning, amongst others, “regulation of metabolic process” ($P=0.0063$; GO:0019222; N=46, e.g. *Calr*, *Igfbp5*, *Sox4* and *Tcf4*), “skeletal system development” ($P=0.00395$; GO:0001501; N=12, e.g. *Ctnnb1*, *Ctgf*, *Sox4* and *Mef2c*), “anatomical structure development” ($P=0.039$; GO:0048856; N=36, e.g. *Sox4*, *Notch3*, *Egfr*, *Ctnnb1* and *Mef2c*), and “regulation of response to stress” ($P=0.0171$; GO:0080134; N=14; *Setd8*, *Ankrd1*, *Mef2c*, *Egfr* and *Ctgf*) with application of a false discovery rate algorithm.

To connect thyroid hormone signaling with the effects of forced mechanical stress, we added genes involved in intracellular thyroid hormone signaling (*Dio2*, *Thra*, *Thrb* and *Rxra*) into the protein-protein interaction prediction. The network that is formed (Figure 3) shows thyroid signaling genes/proteins to be incorporated into the large network, as previously seen (Figure S1). Thyroid signaling was found to interact directly with the differential expressed genes through, *Ctgf* (A) and *Egfr* (B), via thyroid receptor alpha (*Thra*) and retinoid x receptor (*Rxra*), both known factors in the development of OA[30-32].

Genes differentially expressed in *Dio2*^{-/-} mice compared to wild type mice upon a forced running regime

Since we found differences in differential expression patterns between knockout and wild

type mice upon the forced running regime, we assessed which of the 147 differentially expressed genes (Figure 1 and Supplementary Table S1) showed significant interaction between running regime and genetic background based on nominal P-values. In total 29 probes, representing 29 genes were found to be significantly differentially expressed in knockout-mice as compared to wild type-mice when undergoing a forced running regime (Table 1). The significant differences in effect (beta-values), as a result of the running regime, between the knockout- and the wildtype-group can be divided in 3 sub-groups. Each group shows a different effect-size based on “genotype” for the 29 genes found to show a significant interaction. We identified 4 genes that showed no differential expression upon running in wild-type mice, but are differentially expressed (down-regulated) upon running in *Dio2*^{-/-} mice (Group 1; e.g. *Gnas* and *Rhbdl2*). In total 16 genes only showed differential expression in wild-type mice and not in the *Dio2*^{-/-} mice (Group 2; e.g. *Hmgb2*, *Calr* and *Lbh*) whereas, 9 genes showed significant differential effect-sizes between wild-type and knockout mice, but having the same direction of effect (Group 3; e.g. *Sox4* and *Socs2*). Depending on the gene-expression pattern the genes in the different groups could mark a favorable effect in the *Dio2*-knockout mice (Group 1) or an unfavorable effect in wild type cartilage homeostasis (Group 2).

Discussion

Dio2-deficient mice showed less cartilage damage and reduced severity of synovitis in a treadmill running model of OA. The absence of significant differential gene expression between the running and no-exercise group in *Dio2*^{-/-} mice suggests that degenerative pathways are not activated in this knockout strain despite the biomechanical burden that is imposed. These data provide novel support for inhibition of *DIO2* as a therapeutic strategy in OA, in particular since no striking developmental skeletal phenotype appears present in the *Dio2*^{-/-} mice (results not shown). Upon forced mechanical loading, wild type mice showed clear signs of OA and differential expression of genes associated with the disease, supporting the use of forced running as an OA-model in mice. A subset of the genes was found directly interacting with thyroid signaling through *Thra*, *Rxra* and *Dio2*, depicted in Figure 3. This indicates the importance of thyroid hormone signaling as a regulatory system in the response to stress and, when suppressed, for the maintenance of cartilage homeostasis.

With the differentially expressed genes in the wild-type stratum and the combined knockout/wild-type stratum, pathway enrichment was found for expected biological processes, such as “skeletal system development”, but also for processes involved in “regulation of response to stress”. Notably, genes overlapping between these enriched processes as well as being ‘nodes’ in the recognized protein interaction networks (*Egfr* (B) and *Ctnnb1*(C); Figure 3) are well known for their association with OA [30, 33, 34]. These could, therefore, point at important modulators affecting the propensity to develop OA upon mechanical stress and as such at potential drugable targets for novel therapeutic approaches.

Tabel 1: Genes that show significant differential expression upon the running regime between wild-type- and knockout-mice (interaction)

Symbol	Run vs NoRun in Knockout			Run vs NoRun in Wildtype			Interaction
	Beta ¹	St. Error ²	P-value ³	Beta	St. Error	P-value	P-value ⁴
Group 1: Genes with an effect of the running regime in knockout- but not in wild-type-mice							
Hist1h2an	-1,024	0,2603	0,000	-0,186	0,1088	0,087	0,0030
Gnas	-0,956	0,2318	0,000	-0,258	0,1473	0,080	0,0111
Rhbdl2	-1,135	0,2176	0,000	-0,349	0,2638	0,185	0,0215
E130112E08Rik	-1,041	0,2981	0,000	-0,26	0,2171	0,231	0,0343
Group 2: Genes with an effect of the running regime in wild-type- but not in knockout-mice							
Anxa11	0,02	0,0852	0,818	-0,636	0,0668	0,000	0,0000
Setd8	-0,006	0,0864	0,943	0,725	0,1044	0,000	0,0000
Phdla1	-0,152	0,1019	0,136	-0,591	0,1011	0,000	0,0022
Hmgb2	0,131	0,1439	0,363	0,666	0,1476	0,000	0,0095
Calr	-0,086	0,1561	0,581	-0,664	0,1658	0,000	0,0111
Gdi1	0,313	0,2297	0,173	0,923	0,1446	0,000	0,0247
Lbh	-0,13	0,0682	0,057	-0,889	0,0761	0,000	0,0000
Fmo5	-0,028	0,1028	0,784	-0,622	0,0848	0,000	0,0000
Igsf4a	-0,171	0,0978	0,081	-0,789	0,1221	0,000	0,0001
Fcho1	0,132	0,1144	0,250	0,665	0,1106	0,000	0,0008
Serpinb1a	0,396	0,3687	0,283	-0,877	0,1145	0,000	0,0010
Mtpn	-0,082	0,1084	0,451	-0,594	0,1121	0,000	0,0010
Rbm47	-0,09	0,075	0,229	-0,592	0,1011	0,000	0,0013
LOC433464	-0,086	0,1638	0,599	-0,675	0,0788	0,000	0,0015
Rer1	-0,185	0,1714	0,281	-0,611	0,1121	0,000	0,0195
Hist1h4i	0,113	0,2763	0,684	-0,811	0,1011	0,000	0,0214
Group 3: Genes with an effect of the running regime in both wild-type- and knockout-mice							
Mll5	-1,378	0,2306	0,000	-0,439	0,1184	0,000	0,0003
Chd1	-1,178	0,2311	0,000	-0,307	0,1125	0,006	0,0007
Septin11	-0,28	0,0887	0,002	-0,671	0,0918	0,000	0,0022
Sox4	-0,479	0,1399	0,001	-0,969	0,1064	0,000	0,0053
Gins1	0,771	0,305	0,011	1,596	0,0735	0,000	0,0085
Socs2	-0,337	0,1132	0,003	-0,713	0,0881	0,000	0,0088
C330023M02Rik	-0,308	0,1498	0,040	-0,678	0,054	0,000	0,0202
Hgsnat	-0,29	0,1274	0,023	-0,621	0,1001	0,000	0,0410
Rap2c	-0,385	0,0743	0,000	-0,628	0,0955	0,000	0,0449

¹The beta-value is representing the difference in fold change between the running group and the non-running group. The direction of the beta-value is how the running-group is different from the non-running group. ²The standard error is showing the variation of the beta-value between samples.

³The nominal P-value is the P-value of that the single probe that was calculated to tell whether based on this single probe, the expression levels between runners is different of that of non-runners.

⁴The Interaction P-value is a summarizing value that tells whether the difference caused by exercise is significantly different between the knockout- and the wild-type-stratum

In contrast, when assessing gene expression in the knockout-group alone, we found no significant differential gene expression upon severe mechanical loading, after multiple testing adjustment. Taken together with the pathology observations these data indicate that the repression of *Dio2* is beneficial against the development of cartilage damage upon mechanical stress. This effect could theoretically also be caused by a difference in power between the different strata (wild-type and knockout). However, since the comparison made in knockout animals (6 running versus 3 controls) is comparable to the wildtype animals (8 running versus 3 controls), this is unlikely to explain the complete absence of a significant differentially expressed gene in the knockout stratum.

Looking at the effect of *Dio2*-deficiency on gene expression, we found that the gene expression response upon exercise between wild-type and knockout-mice was significantly different for 29 genes with nominal significance, of the total set of 147 differentially expressed genes. Genes categorized in group 1 (Table 1), showing no differential expression upon forced running in wild-type mice, but are differentially expressed with nominal significance in *Dio2*^{-/-} mice (*E130112E08Rik*, *Gnas*, *Hist1h2an* and *Rhbdl2*) could represent the respective favorable effects. *Gnas*, for example, is involved in skeletal development (GO:0001501) and complex skeletal disorders such as Albright Hereditary Osteodystrophy that can lead to early OA [35]. *Gnas* upregulation was found directly regulating hypertrophic differentiation of growth plate cartilage *in vivo* [36]. The knockout-specific down-regulation of *Gnas* upon forced running could be a protective mechanism within the *Dio2* knockout mouse.

We hypothesize that the sixteen genes within group 2, only showing differential expression in wild-type mice and not in the knockout-group (e.g. *Hmgb2* and *Calr*), represent the unfavorable, damaging effects of forced running in wild type mice. *Hmgb2* was shown to be expressed at higher levels in human MSCs as compared to human articular chondrocytes and the expression declined during chondrogenic differentiation of MSCs [37]. The up-regulation of *Hmgb2* seen in wild type-mice upon forced running could be explained as a marker that changes in the differentiation status of the chondrocytes are occurring. A second interesting member of this group is calreticulin (*Calr*). *Calr* interacts with the glucocorticoid receptor (GR). It may also interact with other steroid receptors or thyroid receptors in a similar way [38]. Furthermore, *Calr* was shown to be involved in cartilage thinning of mandibular cartilage in a rat model that studied the effects of compressive mechanical loading [39]. In our forced running experiment we see a similar induction of *Calr* expression upon stress, but not in the *Dio2* knockout-mice, possibly giving the knockout-mouse an advantage against the formation of OA-like degradation or cartilage thinning.

Nine additional genes showed significant differential effect-sizes between wild-type and knockout mice, albeit the same direction of effect (e.g. *Sox4* and *Socs2*). Notable within this third group (Table 1) is *Sox4*, which was previously found to be expressed very early during chondrogenesis [40], much earlier than the well defined *Sox5* and *Sox6* [41]. Furthermore, it was shown that expression of *Sox4* could be stimulated by adding physiological concentrations of human parathyroid hormone (PTH), indicating involvement of the PTH/PTHrP receptor [42]. Here we show that *Sox4* expression is influenced by the ab-

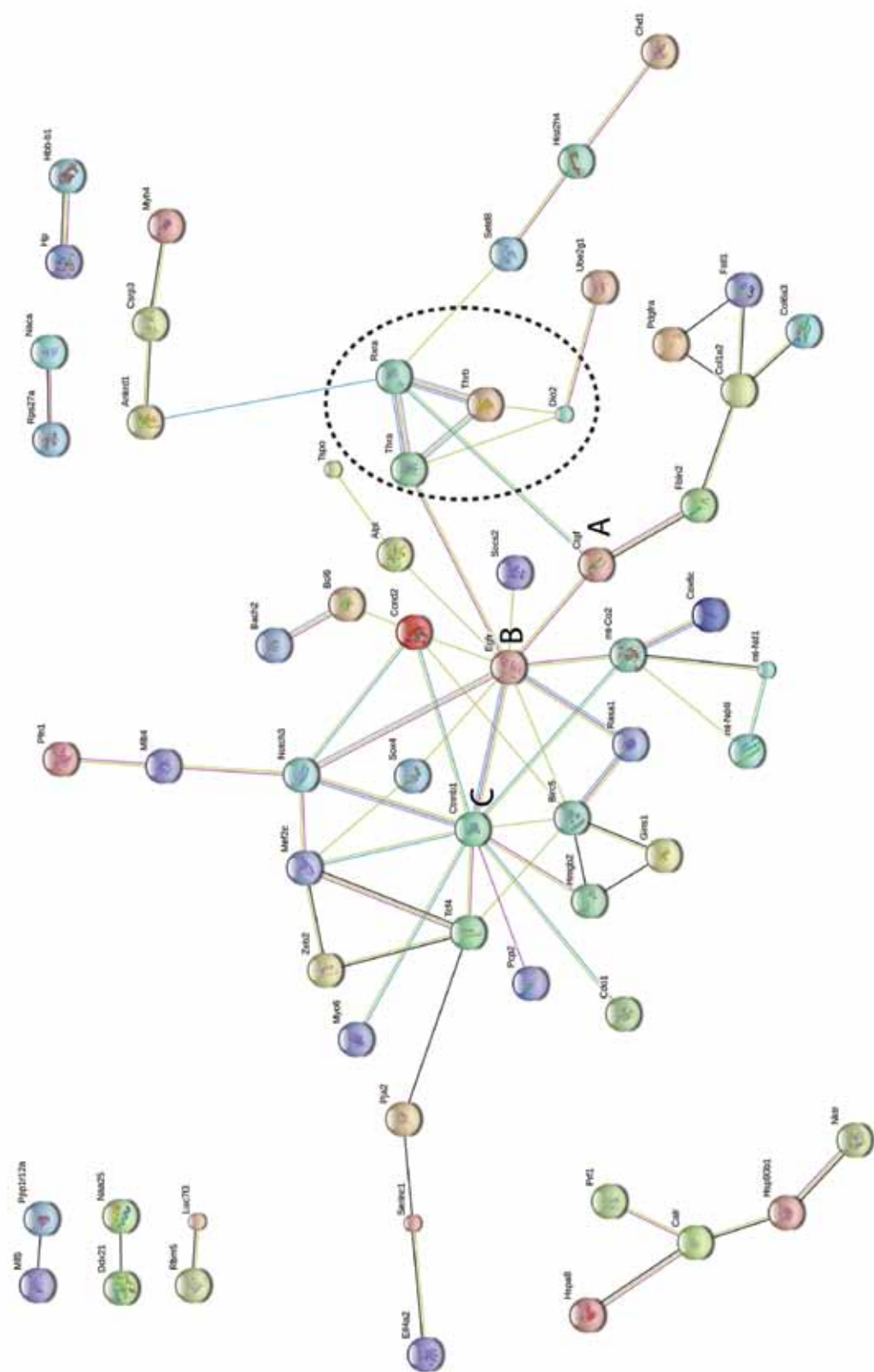


Figure 3. Gene networks in search tool for the retrieval of interacting genes.

Screenshot of the protein–protein interactions (STRING-db) for the 147 genes differing >1.5-fold between ‘runners’ and controls, including thyroid signaling members; *Dio2*, *Thra*, *Thrb* and *Rxra* (encircled by the dashed line). Mayor hubs in the network are depicted by A) *ctgf*, B) *egfr*, and C) *ctnnb1*. Disconnected proteins are hidden.

sence of *Dio2*. In the knockout-mice the expression of *Sox4* is 2 times less down-regulated upon running when compared with the control group. How this influences the structural integrity of articular cartilage remains unknown. Other genes in the list have no straight-forward connection to OA based on current literature.

In conclusion, in the current paper we find that *Dio2* deficiency has a protective effect on the homeostasis of articular cartilage in the knee-joints of mice undergoing a forced running regime. This is consistent with our earlier findings, showing that pharmacological inhibition of deiodinases in a human *in vitro* chondrogenesis model has a beneficial effect on the early formation and maintenance of articular cartilage ECM [18]. It is therefore hypothesized that control of thyroid hormone signaling, both during development and adult cartilage maintenance, is essential to ensure normal bone and cartilage homeostasis, and that it could act as the master-switch that forces maturational arrested chondrocytes to re-activate the endochondral ossification process, leading to articular cartilage destruction. Our results show that interfering with intracellular thyroid hormone levels could be a powerful way to oppose the pathological events that are occurring in OA.

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Competing interest

The authors declare no competing financial, personal, or professional interests.

Author contributions

Conceived and designed the experiments: NB; FMFC; PES; IM; RJLL

Performed the experiments: NB; FMFC; LS; RvdB; NL;

Analyzed the data: NB; FMFC; YFMR; WdH; IM; RJLL

Wrote the manuscript: NB; FMFC; IM; RJLL

Critically reviewed the manuscript: All authors

Supplemental Data

<http://ard.bmj.com/content/75/3/571/suppl/DC1>

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Chapter 5

Aberrant Calreticulin expression in articular cartilage of *Dio2* deficient mice

Nils Bomer^{1,*}, Frederique M.F. Cornelis², Yolande F.M. Ramos¹, Wouter den Hollander¹, Nico Lakenberg¹, Ruud van der Breggen¹, Lies Storms⁴, P. Eline Slagboom¹, Rik J.U. Lories^{2,3}, Ingrid Meulenbelt¹

¹Dept. of Molecular Epidemiology, LUMC, Leiden, Netherlands; ²Laboratory of Tissue Homeostasis and Disease, Skeletal Biology and Engineering Research Centre, KU Leuven, Leuven, Belgium; ³Division of Rheumatology, University Hospitals Leuven, Leuven, Belgium

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Abstract

Objective: To identify intrinsic differences in cartilage gene expression profiles between wild-type- and *Dio2*^{-/-}-mice, as a mechanism to investigate factors that contribute to prolonged healthy tissue homeostasis.

Methods: Previously generated microarray-data (Illumina MouseWG-6 v2) of knee cartilage of wild-type and *Dio2*^{-/-}-mice were re-analyzed to identify differential expressed genes independent of mechanical loading conditions by forced treadmill-running. RT-qPCR and western blot analyses of overexpression and knockdown of *Calr* in mouse chondro-progenitor cells (ATDC5) were applied to assess the direct effect of differential *Calr* expression on cartilage deposition.

Results: Differential expression analyses of articular cartilage of *Dio2*^{-/-} (N = 9) and wild-type-mice (N = 11) while applying a cutoff threshold ($P < 0.05$ (FDR) and $FC > |1.5|$) resulted in 1 probe located in Calreticulin (*Calr*) that was found significantly downregulated in *Dio2*^{-/-} mice ($FC = -1.731$; $P = 0.044$). Furthermore, overexpression of *Calr* during early chondrogenesis in ATDC5 cells leads to decreased proteoglycan deposition and corresponding lower Aggrecan expression, whereas knocking down *Calr* expression does not lead to histological differences of matrix composition.

Conclusion: We here demonstrate that the beneficial homeostatic state of articular cartilage in *Dio2*^{-/-} mice is accompanied with significant lower expression of *Calr*. Functional analyses further showed that upregulation of *Calr* expression could act as an initiator of cartilage destruction. The consistent association between *Calr* and *Dio2* expression suggests that enhanced expression of these genes facilitate detrimental effects on cartilage integrity.

Introduction

Osteoarthritis (OA) is a prevalent, complex, chronic and disabling disease among elderly and is characterized by progressive destruction of joint cartilage, remodeling of the subchondral bone, formation of osteophytes and synovitis [1,2]. The aetiology of OA is not entirely understood, yet multiple factors such as joint injury, obesity and mechanical stress have been found to significantly contribute to the onset and progression of the disease. Moreover, OA has a considerable genetic component and a variety of genetic studies have identified multiple genes that robustly confer risk to OA [3,4]. A notable OA risk gene is the deiodinase iodothyronine type-2 (D2) gene (*DIO2*) [5] that has multiple lines of substantiating functional follow up data indicating that risk allele carriers have a more vivid upregulation of *DIO2* expression in articular cartilage. *In vitro* cell studies as well as a cartilage-specific transgenic rat study indicated that this resulted in cartilage destruction. In this *in vitro* model, inhibiting *DIO2* action, resulted in prolonged cartilage homeostasis, as where *Dio2*^{-/-}-mice were shown to be protected against treadmill-running induced OA [6]. Moreover, genome wide expression analysis of articular cartilage of *Dio2*^{-/-} and wild-type mice, before and after forced treadmill-running, demonstrated that particularly *Calr*, *Hmgb2*, *Sox4* and *Socs2* were differentially regulated in the *Dio2*^{-/-} mice upon the mechanical applied stresses [6]. As *Dio2*^{-/-} mice had less cartilage damage these genes likely contribute to enhanced adaptive capacity to environmental challenges such as mechanical overloading. In parallel to these finding, there is need for insight into intrinsic molecular pathways that assure articular cartilage tissue homeostasis independent of environmental challenges. In the current study, we therefore re-analysed our micro-array expression data of wild type and *Dio2*^{-/-} mice independent of mechanical loading by treadmill-running and performed functional studies to validate results.

Materials and methods

Dio2^{-/-} treadmill-running animal model and microarray assay

Animal experiments

As described previously [6], *Dio2*^{-/-}-mice were a kind gift of Dr. V. Galton (Dartmouth Medical School, NH, USA) and were backcrossed onto the C57Bl/6 background. In the experiments reported here, mice were in the 6th generation of backcrossing. Wild-type C57/Bl6 mice were purchased from Janvier (Le Genest St Isle, France). All experiments were approved by the Ethics Committee for Animal Research (KU Leuven, Belgium).

Four to 6-months old male *Dio2*^{-/-} and wild-type-mice were subjected to a forced treadmill-running regimen on a four lane modular treadmill (Columbus Instruments, Columbus, OH, USA). These mice ran for 3 weeks 1 hour/day, 5 days/week, at a speed of 11 m/min and with an inclination of 5°. All animals, including mice that were used as non-running controls, were littermates and were caged together and housed in the same facility [6]. Mice were group housed in standard mouse cages with sawdust as bedding material

and under conventional laboratory conditions; constant room temperature (22 +/- 2 °C), humidity level (55 +/- 5 %), a 12-h light:12-h dark cycle (lights on at 8 AM) and standard food (Sniff, Soest, Germany) and water available ad libitum. Mice were euthanized by decapitation under sedation.

RNA isolation of murine knee cartilage

For gene expression studies, cartilage was micro-dissected, snap frozen in liquid nitrogen, and stored at -80° Celsius upon isolation. Cartilage of left-side knee-joints of *Dio2^{+/-}* (n=6 NoRun and n=12 Run) and wild-type-mice (n=6 NoRun and n=16 Run) was used for RNA isolation. To gain isolation efficiency and thereby mRNA quality, articular cartilage of knee-joints of two mice from the same sub-group were pooled resulting in n=9 *Dio2^{+/-}* and n=11 wild-type samples, analyzed in the manuscript [6].

Microarray analysis

Sample homogenization, RNA isolations, complementary DNA synthesis, amplification, biotin labeling and hybridization onto the Illumina MouseWG-6 v2 BeadChip microarrays (Illumina, Eindhoven, The Netherlands) were performed as described [6]. Samples were dispersed for experimental condition over the arrays of 6 chips from a single batch, and each chip contained a replicate sample that was also present on a second chip, to exclude batch effects across chips. Slide scanning, basic quality control and data normalization were performed as described [6]. Replicates measured over the different chips were taken into account when performing normalization. For further analysis, the replicate with the lowest number of calls was discarded from the dataset. Raw probe-level data were exported for analyses using the Limma R-package [7]. We checked the data for large-scale batch effects between chips using principal component analysis. Probes with P-value ≤0.05 after FDR correction for multiple testing were considered significant.

Gain and loss of function of *Calr* in ATDC5, chondrogenic induction and histological evaluation

The murine chondrogenic cell line ATDC5 (kindly provided by H.C.M. Sips, LUMC) was maintained in DMEM/Ham's F-12 (1:1 Gibco) supplemented with 5% FBS and antibiotics at 37°C in a humidified 10% CO₂ / 90% atmosphere. To induce chondrogenesis, ATDC5 cells were plated in the medium described above supplemented with ITS+ (6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 ng/ml sodium selenite, 5.33 linoleic acid, and 1.25 µg/ml bovine serum albumine; Becton&Dickinson, Breda, The Netherlands). The medium was replaced every other day. The total cell culture time was 4 days.

For *Calr* overexpression purposes, a plasmid vector containing untagged mouse *Calr* (pCMV3-*mCalr*) was purchased (Sino Biological Inc., Beijing, P.R. China). As a control for the *Calr* overexpression experiments, ATDC5 cells were transfected with an empty pCMV3 vector. For knockdown of *Calr* expression, 3 unique 27mer siRNA duplexes, specific for *Calr*, were purchased (OriGene technologies, Rockville, MD, USA). Together with the 27mer siRNA duplexes a universal scrambled negative control siRNA duplex (OriGene technologies) was purchased to be used as a control for the knockdown experiments.

The day before transfection, ATDC5 cells were passaged 1:6 from a confluent 6-well plate into a new 12-wells plate. Fugene® 6 transfection reagent was used to transfect ATDC5 cells with either the *mCalr*-vector or control vector, or the mixed siRNA duplexes or control duplex, according to the manufacturers protocol. One day after transfection, regular proliferation medium was changed for chondrogenesis medium. After 4 days of chondrogenesis, the cells were harvested for RNA and histology.

ATDC5 cells were fixed in 4% formaldehyde and stained for acidic polysaccharides (glycosaminoglycans (GAGs)) with Alcian Blue (8-GX pH=1; Sigma-Aldrich). To measure the staining intensity, the Alcian Blue staining was washed with 6M guanidine hydrochloride (Sigma-Aldrich) to decolorize the extracellular matrix. Intensity of the supernatant was measured using a photospectrometer at 620 nm wavelength. Experiments were performed with biological triplo's.

DIO2 overexpressing hBMSCs, 3D chondrogenic induction and mRNA evaluation

Previously, we performed *in vitro* 3D chondrogenesis using lentiviral DIO2 transduced hBMSCs [8]. Chondrogenesis was initiated in 0.5 ml serum-free chondrogenic differentiation medium (DMEM, supplemented with Ascorbic acid (50 µg/ml; Sigma-Aldrich; Zwijndrecht, The Netherlands), L-Proline (40 µg/ml; Sigma-Aldrich), Sodium Puryvate (100 µg/ml; Sigma-Aldrich), Dexamethasone (0,1 µM; Sigma-Aldrich), ITS+, antibiotics, and TGF-β1 (10 ng/ml; PeproTech)). Lentiviral constructs containing C-terminal FLAG-tagged cys-D2 (kindly provided by Prof. Dr. Bianco[9]) were constructed and transduced into hBMSCs as described before [8]. Here we used 3D pellets that were snap frozen in liquid nitrogen and stored at -80 degrees Celsius.

RNA isolation

RNA from the ATDC5 cells was isolated by lysing the cells in 500 µl of TRIzol® reagent (Life Technologies, Bleiswijk, The Netherlands), and 200 µl of chloroform was added before centrifugation (15 min at 14.000g). After addition of 1 v/v 70% ethanol/DEPC-treated water to the aqueous upper layer total RNA was isolated using Qiagen RNeasy mini columns following the manufacturer's protocol. RNA isolation from the hBMSC pellets was performed by pooling two pellets for every given condition, respectively. The isolation of RNA was performed as described previously[10]. RNA quantity was assessed using a nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA).

Quantitative RT-PCR assay (validation)

Isolation of RNA was performed as described previously[6]. Approximately, 500 ng of total RNA was processed with the "First Strand cDNA Synthesis Kit" according to the manufacturer's protocol (Roche Applied Science, Almere, The Netherlands), upon which cDNA was diluted 5 times. RT-qPCR measurements were performed on the Roche Lightcycler 480 II, using Fast Start Sybr Green Master reaction mix according to the manufacturer's protocol (Roche Applied Science). Relative gene expressions of the Roche Lightcycler 480 II data were calculated by using the $2^{-\Delta\Delta Ct}$ method[11]. The housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a single refer-

ence gene for qPCR[12,13]. Primer efficiencies were verified by performing a concentration curve experiment (primer sequences used are listed in [S1 Table](#)). The student T-test was used to calculate the significance. All P-values < 0.05 were considered statistically significant.

Results

After quality control and normalization of micro array expression data of articular cartilage of N=9 *Dio2*^{-/-} and N=11 wild-type mice, 18226 of the 45281 probes were found to have a detectable expression level, representing 12312 unique genes (~49% of the mouse transcriptome) and 2707 RIKEN sequences ([S2 Table](#)). Differential expression analyses while applying a cutoff threshold ($P < 0.05$ (FDR) and $FC > |1.5|$)[6] resulted in only 1 probe located in Calreticulin (*Calr*) that was found significantly downregulated in *Dio2*^{-/-} mice ($FC = -1.731$; $P = 0.044$). Technical validation by RT-qPCR in N=20 samples (N=9 *Dio2*^{-/-} and N=11 wild type mice) of the discovery cohort confirmed the expression difference ([S1 Figure](#)). Notably, our previous data showed that forced treadmill-running had a profound effect on *Calr* expression ($FC = -1.58$, $P = 0.0418$)[6] in cartilage of wild-type-mice. However, stratifying expression of *Calr* by both mice type (*Dio2*^{-/-}, Wildtype) and exposure (Run, Norun) shows that the here identified *Calr*-effect appears to be independent of the mechanical challenge ([Figure 1](#)).

Next, we investigated the effect of altered *Calr* expression on the chondrogenic capacity of ATDC5 cells. As measured by photospectrometry, overexpressing *Calr* during early 2D chondrogenesis resulted in significant lower Alcian Blue staining, indicating less glycosaminoglycan deposition ([Figure 2](#)). Concurrently, RT-qPCR confirmed significant lower mRNA expression levels of *Acan* ($FC = -2.32$) ([Figure 3](#)). Of note was the 1.8-fold upregulation of *Dio2* in the cells overexpressing *Calr*, albeit that this effect by definition was not significant ($P = 0.08$). On the other hand, knockdown of *Calr*, by siRNA duplexes in this model, did not show an effect on the amount of deposited glycosaminoglycans stained by Alcian Blue ([Figure 2](#)) nor on the mRNA expression levels of *Acan* ([Figure 3](#)). However, a significant 4.7-fold downregulated effect was observed for *Dio2* mRNA expression levels ($P = 0.017$).

Finally, to confirm the transcriptional link between of *DIO2* and *CALR* expression, we examined *CALR* expression in cartilage constructs previously generated from human BM-SCs *in vitro* 3D chondrogenesis and compared them to those generated while applying overexpression of *DIO2* by lentiviral transduction[8]. As shown in [Figure 4](#), also in this human BMSCs *in vitro* 3D chondrogenesis model overexpressing *DIO2* coincided with significant higher *CALR* expression.

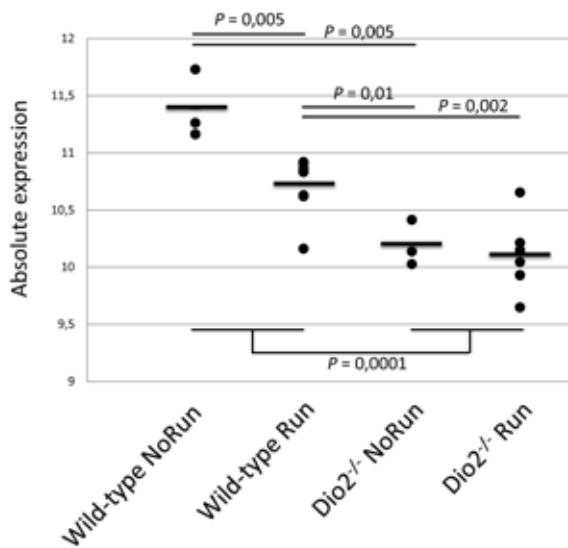


Figure 1. Calreticulin expression stratified for treadmill-running and knockout.

Representation of the *Calr* expression values of all individual samples plotted by genotype-group (Wild-type and *Dio2*^{-/-}) and treadmill-running-group (No Run and Run). Showing the significant reduction of *Calr* expression upon forced treadmill-running in wild-type-mice ($P = 0,005$) and the absence of change in the knockout-mice. P-values depicted are derived from Student T-Test.

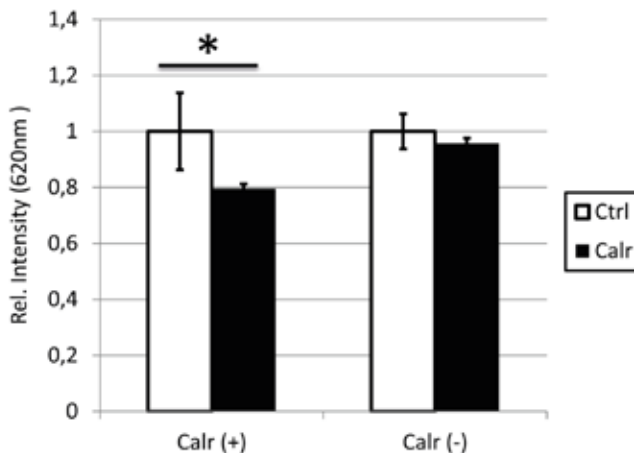


Figure 2. Overexpressing *Calr* resulted in significantly lower Alcian Blue (AB) staining intensities.

Staining intensities of Alcian Blue staining measured with a photospectrometer (620 nm). Values are displayed as the average $\pm$ SEM, relative to the control sample. Differences were analyzed with Student T-Test ((*) $P < 0.05$).

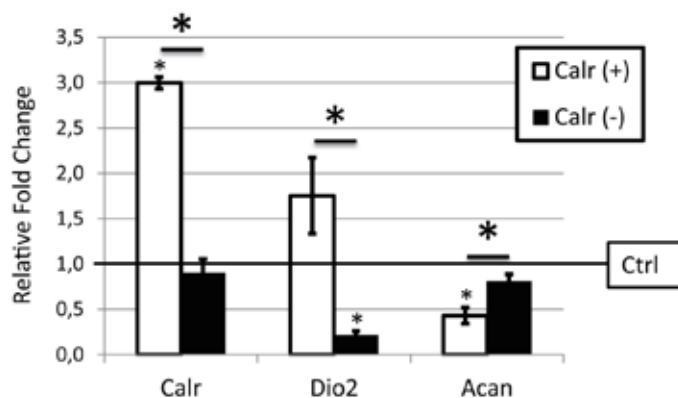


Figure 3. Expression analyses in *Calr* overexpressing ATDC5 cells.

RT-qPCR expression of *Calr*, *Dio2* and *Acan* in ATDC5 cells transfected with either a *Calr*-containing vector for overexpression (*Calr*+) or 3 unique 27mer siRNA duplexes, specific for *Calr*, for knock-down (*Calr*-). Values of the RT-qPCR are displayed as the average $\pm$ SEM, normalized for *GAPDH* expression and relative to the control samples (dashed line). Differences were analyzed with Student T-Test (*) $P < 0.05$.

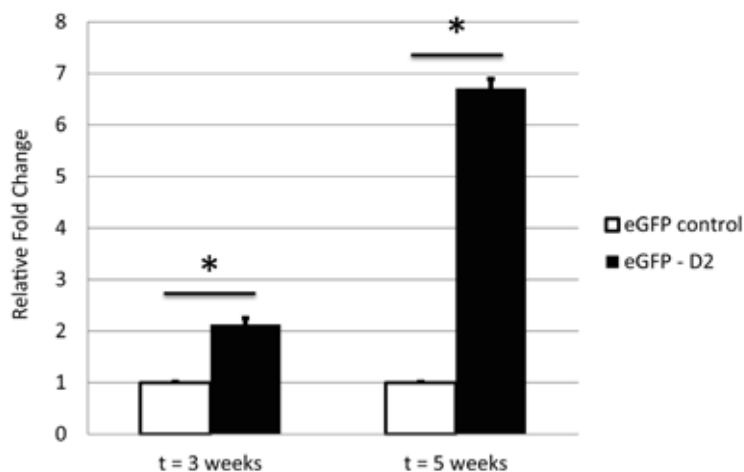


Figure 4. Calreticulin expression in *DIO2* overexpressing hBMSCs.

qPCR expression of *Calr* in hBMSCs transduced with either a control virus-vector (eGFP) or a *DIO2*-eGFP vector. Values of the RT-qPCR are displayed as the average $\pm$ SEM, normalized for *GAPDH* expression and relative to the control sample at every timepoint. Differences were analyzed with Student T-Test (*) $P < 0.05$.

Discussion

In parallel to disease modifiers, there is a need for insight into intrinsic factors that enhance stability of healthy articular chondrocytes. We show that cartilage of the *Dio2*^{-/-} as compared to wild type mice has an intrinsic difference in *Calr* expression. Since *Dio2*^{-/-} mice were previously found to be less susceptible to mechanically induced damage, our data indicate that mitigated *Calr* and/or *Dio2* expression, mark a beneficial homeostatic state of articular cartilage. Despite the, here outlined, consistent association between *Calr* and *Dio2* expression, no thyroid responsive elements were identified near *Calr*[14] nor did *Calr* show any predicted interactivity with thyroid hormone signaling based on String-DB [15].

Calr is a multifunctional protein that was shown to have many putative roles. Some examples are: Ca²⁺-binding, storage and stress induced release[16], quality control molecular chaperone binding to miss folded proteins and preventing them from being exported from the endoplasmic reticulum to the Golgi apparatus, and modulator of steroid-sensitive gene expression[17–21]. For that matter, the amino terminus of *Calr* interacts with the DNA-binding domain of nuclear hormone receptors (the glucocorticoid receptor[17], androgen receptor[18], and retinoic acid receptor[22]) and prevents the receptors from binding to their specific response elements, influencing transcriptional activities *in vitro* and *in vivo*. Furthermore, *Calr* expression was shown to be associated with cartilage thinning of mandibular cartilage in a rat model that studied the effects of compressive mechanical loading[16], and has been implicated in the pathogenesis of rheumatoid arthritis (RA)[23].

Similarly in this respect, we showed that overexpression of *Calr* during early chondrogenesis in ATDC5 cells leads to decreased proteoglycan deposition and corresponding lower Aggrecan expression, whereas knocking down *Calr* expression does not lead to histological differences of matrix composition. It was shown before that increasing levels of *Calr*, sensitized the cells to apoptosis during mechanical-induced endoplasmic reticulum-stress, while decreasing *Calr* levels protected cells from apoptosis[24]. It was hypothesised that by regulating the amount of calcium stored in the ER, and therefore the amount that can be released to the cytosol to trigger downstream events, *Calr* can affect apoptotic outcomes[25]. Given our data, we hypothesize that lower levels of *Calr* expression, as seen in the *Dio2*^{-/-}-mice, could results in a more subtle calcium-induced apoptotic signal upon stress, being favourable for the maintenance of cartilage tissue homeostasis. The beneficial effect of reduced calcium-levels for cartilage integrity is supported by a recent study which reported that calcium antagonists might be efficient in preventing progression of OA[26].

Together, we here demonstrate that the beneficial homeostatic state of articular cartilage in *Dio2*^{-/-} mice is accompanied with significant lower expression of *Calr*. Moreover, we showed that increased *Calr* expression could directly provoke cartilage destruction. Given their consistent association, we conclude that enhanced interactive expression of *Calr* and *Dio2* contributes to detrimental effects on cartilage integrity. The precise molecular mechanism of *Calr* in the maintenance of cartilage homeostasis and its interplay with *Dio2* signalling is, however, little explored and more research is therefore needed.

Data Availability

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE79239 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE79239>).

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Competing interests

The authors have declared that no competing interests exist.

Author contributions

Conceived and designed the experiments: NB FMFC PES RJUL IM. Performed the experiments: NB FMFC LS RvdB NL. Analyzed the data: NB FMFC YFMR WdH RJUL IM. Contributed reagents/materials/analysis tools: NB FMFC YFMR WdH RJUL IM. Wrote the paper: NB FMFC YFMR WdH NL RvdB LS PES RJUL IM.

Supplemental Data

<http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0154999#sec017>

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Chapter 6

Neo-cartilage engineered from primary chondrocytes is epigenetically similar to autologous cartilage, in contrast to using mesenchymal stem cells

N. Bomer^{1,2}, W. den Hollander¹, H. Suchiman¹, E. Houtman¹, R.C. Slieker^{1,2}, B.T. Heijmans^{1,2}, P.E. Slagboom^{1,2}, R.G.H.H. Nelissen³, Y.F.M. Ramos¹, I. Meulenbelt^{1,*}.

¹Dept. of Molecular Epidemiology, LUMC, Leiden, The Netherlands. ²IDEAL, ³Dept. of Orthopaedics, LUMC, Leiden, The Netherlands.

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Abstract

Objectives: To compare the epigenetic landscape of 3D cell models of human primary articular chondrocytes (hPACs) and human bone-marrow derived mesenchymal stem cells (hBMSCs) and their respective autologous articular cartilage.

Design: Using Illumina Infinium HumanMethylation450 BeadChip arrays, the DNA methylation landscape of the different cell sources and autologous cartilage was determined. Pathway enrichment was analyzed using DAVID.

Results: Principal Component Analysis (PCA) of methylation data revealed separate clustering of hBMSC samples. Between hBMSCs and autologous cartilage 86881 CpGs (20,2%), comprising 3034 differentially methylated regions (DMRs; $\Delta\beta > 0.1$; with the same direction of effect), were significantly differentially methylated. In contrast, between hPACs and autologous cartilage only 5706 CpGs (1,33%) were differentially methylated. Of interest was the finding of the transcriptionally active, hyper-methylation of a Cartilage Intermediate Layer Protein (*CILP*) annotated DMR ($\Delta\beta = 0.16$) in PAC-cartilage, corresponding to a profound decrease in *CILP* expression after *in vitro* culturing of hPACs as compared to autologous cartilage.

Conclusions: In vitro engineered neo-cartilage tissue from primary chondrocytes, hPACs, exhibits a DNA methylation landscape that is almost identical (99% similarity) to autologous cartilage, in contrast to neo-cartilage engineered from bone marrow-derived MSCs. Although hBMSCs are widely used for cartilage engineering purposes the effects of these vast differences on cartilage regeneration and long term consequences of implantation, are not known. The use of hBMSCs or hPACs for future cartilage tissue regeneration purposes should therefore be investigated in more depth in future endeavors to better understand the consequences of the differential methylome on neo-cartilage.

Introduction

Articular cartilage is a highly specialized tissue present in all synovial joints at the ends of longitudinal bones. Its main function is to facilitate protection of subchondral bone against heavy loads, while maintaining smooth locomotor function of the articular joint[1]. Emerging data show that DNA methylation of CpG dinucleotides has a prominent function in both dynamic changes in gene expression and maintaining cellular phenotypes[2-4]. DNA methylation, in which the cytosine residue in cytosine-phosphate-guanine dinucleotides (CpGs) acquires a methyl group, is known to regulate gene expression upon environmental changes such as age and disease. Loss of epigenetic control of articular chondrocytes could be one of the underlying mechanisms preceding disease onset of e.g. osteoarthritis[5]. With age and trauma, articular chondrocytes tend to undergo progressive dedifferentiation[6-8] and degeneration[9-11], resulting in degradation of the cartilage extra cellular matrix (ECM) and subsequent calcification[12], yet without effective treatment. To repair damaged cartilage, regeneration therapies have shown promising results[13-15]. Despite these successes, limitations clearly exist. The shortage of human primary articular chondrocytes (hPACs) and the dedifferentiation encountered in *in vitro* culturing are considered major hurdles. For that matter, mesenchymal stem cells (MSCs) were found to be a promising alternative as candidate cells for cartilage tissue engineering[16]. MSCs can be obtained in large quantities from e.g. adipose tissue and bone marrow and can be expanded for a number of passages without losing their ability to undergo chondrogenic differentiation[17, 18]. Unfortunately, the phenotype of MSCs in cartilage repair is unstable[19]. The expression of cartilage hypertrophy markers by MSCs undergoing chondrogenesis, raises concerns for clinical applications of MSC-based tissue engineering, since chondrocyte hypertrophy in neo-cartilage could ultimately lead to apoptosis and ossification[20, 21]. The exact molecular mechanism underlying hypertrophic differentiation is not understood and despite numerous studies about the function of single signaling pathways in hypertrophy, studies which explore comprehensive pathways in hypertrophic differentiation of MSCs and chondrocytes have not been published in recent years[22]. Moreover, these studies have, until now, not looked at the genome wide DNA methylome which is a crucial regulator of cellular identity and characterizes tissue homeostasis. Overall differences in the methylome on the tissue level are commonly reflected in differentially methylated regions (DMRs), whereas changes in methylation at single CpGs are found to mark, dynamic responses to environmental challenges but also merely stochastic differences at the individual level.[3, 23] For that matter, both changes in DMRs as well as at CpG-sites are known to modulate transcription factor binding and thereby dynamically regulating gene expression[3, 24, 25]. In this study, we examined the genome-wide DNA methylation profiles of 3D chondrogenic cultured human bone marrow-derived MSCs (hBMSCs) and hPACs, against those of the respective hip and knee autologous cartilage.

Materials and methods

Biobanking and tissue sampling

As part of the ongoing RAAK study, ethical approval was obtained from the medical ethics committee of the LUMC (P08.239) and informed consent was obtained from all participants. Participant details are listed in [Table S1](#). Tissue sampling was performed as described before[26]. In short, within 2 hours following surgery, cartilage was washed extensively with phosphate buffered saline to decrease the risk of contamination with blood. Macroscopically unaffected cartilage was sampled from patients who underwent a total joint replacement due to primary OA of either knee (N=6) or hip (N=5). During collection with a scalpel, care was taken to avoid contamination with bone or synovium. Collected cartilage was snap frozen in liquid nitrogen and stored at -80°C prior to RNA and DNA extraction.

Cell isolation and culture

hBMSCs were isolated from hip joints of 4 OA patients who underwent total hip arthroplasty as result of end stage OA as part of the RAAK study. Proliferation for the quantitative expansion of cells, formation of 3D pellet cultures and subsequent chondrogenic differentiation were performed as described before[27]. In short, hBMSCs were expanded for 5 passages before the formation of pellet cultures in 15 ml polypropylene conical tubes. Pellet cultures were harvested for RNA and DNA isolation purposes at time points 14, 21, 35 and 49 days after the start of differentiation. Micromasses were harvested from 14 days onwards, earlier results showed that around this time point, the cells in the micro-mass-cultures are differentiated towards chondrocytes, capable of depositing cartilage-like extracellular matrix[27] ([Figure S1](#)). For every time point, per donor, two separately grown 3D pellet-cultures were pooled as 1 sample before DNA isolation, resulting in 4 independent samples per timepoint.

hPACs were isolated from cartilage derived from four OA patients who underwent total joint arthroplasty of the knee (RAAK study). Macroscopically unaffected cartilage tissue was incubated overnight in DMEM (high glucose; Gibco, Bleiswijk, The Netherlands) supplemented with 10% fetal bovine serum (FBS; Gibco), antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin; Gibco) and 2 mg/ml collagenase type I at 37 °C in a humidified 5% CO₂ / 95% atmosphere. Subsequently, primary chondrocytes were resuspended and filtered through a 100 µm mesh to remove undigested cartilage fragments and extracellular matrix debris. Cells were expanded at 37 °C in a humidified 5% CO₂ / 95% atmosphere in DMEM supplemented with 10% FBS, penicillin (100 units/mL), streptomycin (100 units/mL) and 0.5 ng/ml FGF-2 (PeproTech, Heerhugowaard, The Netherlands) for 2 passages. 3D pellets were formed using centrifugal forces (1200 rpm; 5 min) on 2.5 x 10⁵ cells in 15 ml polypropylene conical tubes. Chondrogenesis was initiated in 1 ml serum-free chondrogenic differentiation medium (DMEM, supplemented with Ascorbic acid (50 µg/ml; Sigma-Aldrich; Zwijndrecht, The Netherlands), L-Proline (40 µg/ml; Sigma-Aldrich), Sodium Pyruvate (100 µg/ml; Sigma-Aldrich), Dexamethasone (0.1 µM; Sigma-Aldrich), ITS+ (Sigma-Aldrich), antibiotics, and TGF-β1 (10 ng/ml; PeproTech)). Medium was

changed every 3–4 days. Pellet cultures were harvested for RNA and DNA isolation purposes at time points 0, 4, 7, 14 and 21 days after the start of differentiation. 0 days hPACs were included as starting point of harvesting, since these cells are already fully differentiated chondrocytes, capable of depositing cartilage-ECM and therefore more or less represent the state in which the 14 days differentiated hBM-MSCs are. Based on 3D micromass culture experiments using ATDC5 cells[28], we decided to use these timepoints (7, 14 and 21 days) for our experiments, with the addition of an early timepoint (4 days) (Figure S1). For every time point, per donor, two separately grown 3D pellet-cultures were pooled as 1 sample before DNA isolation, resulting in 4 independent samples per timepoint.

DNA isolation

Snap frozen articular cartilage was powderized using a Mixer Mill 200 (Retsch, Germany) with continuous liquid nitrogen cooling. DNA was isolated using the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Methylation arrays

DNA was bisulphite treated using the EZ DNA Methylation kit (Zymo Research Corp, Irvine, CA, USA). DNA methylation was assessed using Infinium HumanMethylation450 BeadChips (Illumina, San Diego, CA, USA). All methylation values are reported as fractions between 0 and 1, commonly known as the β value. Samples were randomly dispersed, while sample pairs were assured to be on the same chip. Raw IDAT files were applied in R statistical language using the *minfi* package[29] and were subjected to standard quality control steps, including bisulphite conversion rate and outlier detection. Ambiguously mapping probes were removed prior to normalization[30]. Next, probes of which over 1% of the samples showed a detection P-value over 0.05 were removed. Probes for which either the green or red channel showed an intensity of 0 were removed as well. In total 32693 probes were removed prior to normalization. Visual inspection of sample clustering was done to detect outliers prior to normalization. Background correction and raw signal normalization (quantile) were performed using the *lumi* package[31], separately for both colour channels. The BMIQ formula was used for correcting probe design bias in the 450k DNA methylation data[32]. Probe level intensities were quantile normalized across samples prior to calculation of the β -values. Using the R package *IlluminaHumanMethylation450k.db*, Illumina identifiers were mapped to the hg19 genome build[33]. CpGs with $P\text{-val} \leq 0.05$ after Bonferroni correction for multiple testing were considered significant.

Principal component analysis and statistical analyses

Principal Component Analysis (PCA) was performed in R on the DNA methylation data to identify putative clusters using an unbiased method. Further statistical analyses were carried out using the *lme4* package. All analyses were performed using linear mixed models (LMMs) and were executed including all timepoints per cell-type. Furthermore, they contained a random effect for patient ID to account for putative correlations and inherent individual differences. Methylation measurements are reported as β -values, reflecting

the fraction of methylation between 0 and 1. Differentially methylated regions (DMRs), stratified for hypo- and hyper-methylated CpGs, were defined by three consecutive significantly differentially methylated CpGs by at least 0.1 $|\Delta\beta|$ and at most 1000 base pairs apart, while allowing for a maximum of three non-significant CpGs[3]. A single DMR can contain CpGs annotated to different genes, therefore, pathway enrichment analysis was performed on all official gene symbols annotated to a DMR by its CpGs. Enrichment analyses were performed using the online annotation tool DAVID[34]. Pathways with P-val ≤ 0.05 after Bonferroni correction for multiple testing were considered significant.

Expression data and t-CpGs

Normalized expression data of articular cartilage from the RAAK study were processed and normalized as described previously (GSE57218)[26]. Normalized methylation data of the articular cartilage samples overlapping the samples used for expression were processed and normalized as described previously[35]. In short, for the 13277 probes (covering 9838 unique genes) that were expressed to detectable extent in articular cartilage, the association with DNA methylation of proximal CpGs was determined. DNA methylation data of CpGs within 10kb of annotated genes was correlated to respective gene expression data of articular cartilage. After multiple testing correction for the number of CpGs for each individual gene, 3748 CpGs were observed that significantly correlated with proximal gene expression, hereafter indicated as transcriptionally active CpGs (t-CpGs)[35].

RNA isolation and RT-qPCR and statistical analysis

Isolation of RNA was performed as described previously[27]. RNA quantity was assessed using a nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA). 500 ng of total RNA was processed with the First Strand cDNA Synthesis Kit according to the manufacturer's protocol (Roche Applied Science, Almere, The Netherlands), upon which cDNA was diluted 5 times. RT-qPCR measurements were performed on the Roche Lightcycler 480 II, using Fast Start Sybr Green Master reaction mix according to the manufacturer's protocol (Roche Applied Science). Relative gene expressions of the Roche Lightcycler 480 II data were calculated by using the $2^{-\Delta\Delta C_t}$ method, in which C_t indicates the fractional cycle number where the fluorescent signal reaches detection threshold[36]. Primer sequences used are listed in [Supplementary Table S2](#). The housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a single reference gene for qPCR[37, 38]. The paired-student T-test was used to calculate the significance of differences in expression between the samples of different origins. All P-values < 0.05 were considered statistically significant.

Results

Principal component analyses (PCA) reveals distinct differences in the epigenetic landscape

Genome wide DNA methylation profiling was performed in all samples, consisting of 3D

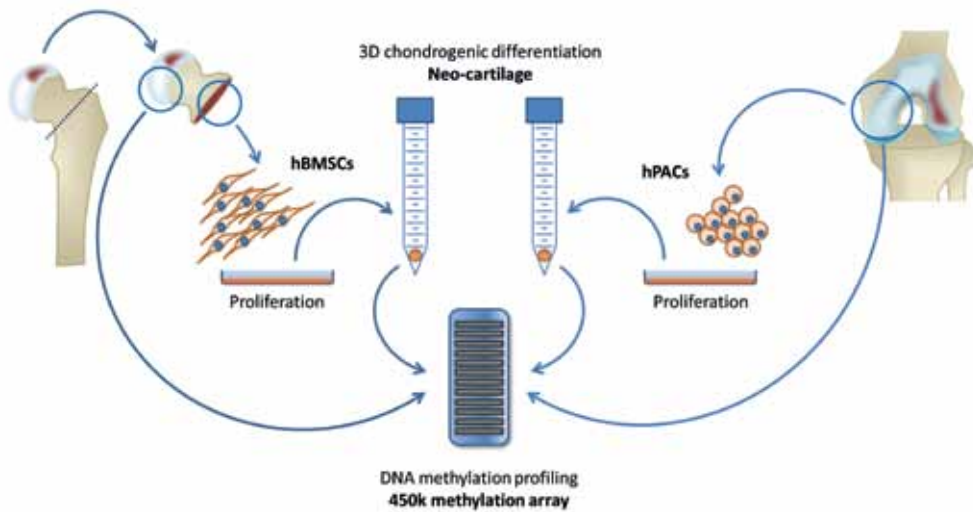


Figure 1: Graphical representation of the study-design.

Human bone marrow derived mesenchymal stem cells (hBMSCs) were isolated from hip joints of OA patients who underwent total hip arthroplasty as result of end stage OA as part of the RAAK study. Primary articular chondrocytes (hPACs) were isolated from macroscopically unaffected cartilage derived from four OA patients who underwent total joint arthroplasty of the knee (RAAK study). Subsequently the cells are proliferated for the expansion of cell-numbers. Next, 3D pellet cultures are formed and chondrogenic differentiation is commenced to engineer hBMSCs-derived neo-cartilage (MSC-cartilage) and hPACs-derived neo-cartilage (PAC-cartilage). Neo-cartilage is isolated for DNA isolation at time points of 14, 21, 35 and 49 days for MSC-cartilage and 0, 4, 7, 14 and 21 days for PAC-cartilage. The genome-wide DNA methylation profiles of MSC-cartilage and PAC-cartilage were compared against those of the respective hip and knee autologous cartilage using Illumina 450k methylation arrays.

chondrogenic cultured hBMSCs derived neo-cartilage (MSC-cartilage; N=4) and hPACs derived neo-cartilage (PAC-cartilage; N=4), and macroscopically unaffected articular cartilage derived from the 4 knee and 4 hip joints from which the cells originated (autologous), as well as 3 additional independent joints (2 knees and 1 hip) (Figure 1). By means of PCA we observed two distinct clusters of samples, being the knee, hip and PAC-cartilage samples together (cluster 1) and the MSC-cartilage separate (cluster 2) on the first component (Figure 2A) indicating that cells in MSC-cartilage samples have a distinct DNA methylation profile compared to autologous cartilage and PAC-cartilage (Figure 2B). On the second PCA component separation of the samples was observed between autologous- and PAC-cartilage (Figure 2A).

The epigenetic landscape of MSC-cartilage is very distinct to autologous cartilage

To elucidate the DNA methylation differences driving the distinct clustering of MSC-cartilage in the PCA, we assessed which CpG-sites were differentially methylated between MSC-cartilage and autologous cartilage (both hip and knee cartilage). We selected CpGs

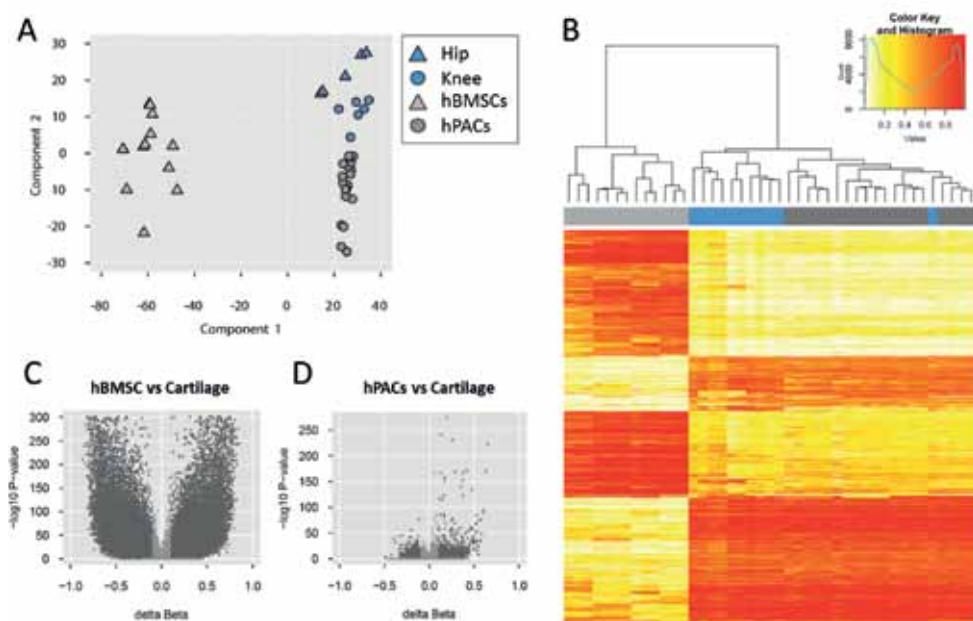


Figure 2. Graphical data representations.

(A) Principle Component Analysis (PCA) displaying the first two components (component 1 (x-axis) and component 2 (y-axis)). (B) Heatmap constructed from the 10000 CpGs (y-axis) that showed the largest variation (Standard Deviation) over the samples (x-axis). Samples are hierarchically clustered (dendrogram on the top), showing two distinct clusters: hBMSCs (light grey) and cartilage (blue) and hPACs (dark grey) together. (C) Volcano-plot of all CpGs after QC (438750) for the comparison of methylation status in hBMSCs against preserved cartilage. CpGs that reach the threshold ($P < 0.05$ & $\Delta\beta > 0.1$) are printed dark grey, CpGs that do not reach the threshold are printed in light grey. (D) Volcano-plot of all CpGs after QC (438750) for the comparison of methylation status in hPACs against preserved cartilage.

that showed significant differential methylation ($P < 0.05$; after adjustment for multiple testing according to Bonferroni) by at least 10% methylation difference ($\Delta\beta$), between MSC-cartilage and paired autologous cartilage (86881 CpGs) independent of the time point of harvest (Figure 2C; Table S3). To prioritize on DMRs, we applied a sliding window algorithm[3] on the differentially methylated CpGs, to distinguish inherent MSC-cartilage differences in the methylation data (DMRs) from possible environmental, stochastic or individual CpG differences (default setting: $\text{DMR} \geq 3\text{CpGs}$). A total of 5884 DMRs were identified, 2855 hypo-methylated DMRs in MSC-cartilage compared to autologous cartilage, and 3029 hyper-methylated DMRs. To be able to perform pathway analyses with DAVID, which allows enrichment analyses for 3000 genes, we raised the threshold for DMR calling from 3 to 4 CpGs. This resulted in 3034 DMRs (1504 hypo-methylated; 1530 hyper-methylated), comprising 16820 CpGs, annotated to 3107 unique transcripts (Table S4), coding for 2575 unique proteins.

Overall pathway enrichment using DAVID revealed enrichment clusters for pathways

Table 1. Summary of Annotation clustering results from DAVID analysis of unique gene symbols annotated to the DMRs in hBMSCs

	Category	Term	Count	Bonferroni
Annotation Cluster 1 <i>Enrichment Score: 13,53</i>	INTERPRO	IPR001356:Homeobox	72	1,88E-10
Annotation Cluster 2 <i>Enrichment Score: 8,07</i>	GOTERM_BP_FAT	GO:0048598~embryonic morphogenesis	92	1,35E-12
Annotation Cluster 3 <i>Enrichment Score: 7,52</i>	GOTERM_BP_FAT	GO:0007389~pattern specification process	67	0,000071
Annotation Cluster 5 <i>Enrichment Score: 7,13</i>	GOTERM_BP_FAT	GO:0060173~limb development	35	0,00014
Annotation Cluster 7 <i>Enrichment Score: 5,51</i>	GOTERM_BP_FAT	GO:0048705~skeletal system morphogenesis	34	0,0045
Annotation Cluster 8 <i>Enrichment Score: 4,63</i>	GOTERM_BP_FAT	GO:0060429~epithelium development	57	0,00091
Annotation Cluster 18 <i>Enrichment Score: 2,73</i>	GOTERM_BP_FAT	GO:0030182~neuron differentiation	89	0,0087

involved in various developmental and transcription regulation processes (GO- and INTERPRO-terms). Amongst others, processes known to be involved in skeletal development and morphogenesis, like: homeobox, embryonic morphogenesis, pattern specification process, limb development and skeletal system morphogenesis (Table 1). But also pathways less recognizable for cartilage development, such as neuron differentiation and epithelium development (Table 1).

Notably, DMRs of the counted genes of Table 1, appeared to be marked by large methylation differences ($\Delta\beta > 0.5$), as such potentially affecting respective transcriptional activity[3, 25, 35]. These are genes known to be important in de early development of the skeleton (e.g. *LRP5*, *COL11A2*, *MSX1*, *GLI3*, *WNT5A* and *TWIST1*), but also neuronal development (*ISL1*, *DLX2*, *IRX3* and *DLX1*). To assess the potential transcriptional consequences of these CpG sites, we examined correlation between methylation and transcription of these genes (*in silico*) in a previously assessed dataset of articular cartilage[35]. Methylation of N= 65 out of N= 583 CpG sites appear to show strong correlation with expression in articular cartilage (Figure 3A-E)[35].

The epigenetic landscape of PAC-cartilage is very similar to analogous cartilage

To identify which genes/processes are epigenetically differently regulated in hPACs proliferated in 2D and cultured under 3D chondrogenic conditions (PAC-cartilage), as com-

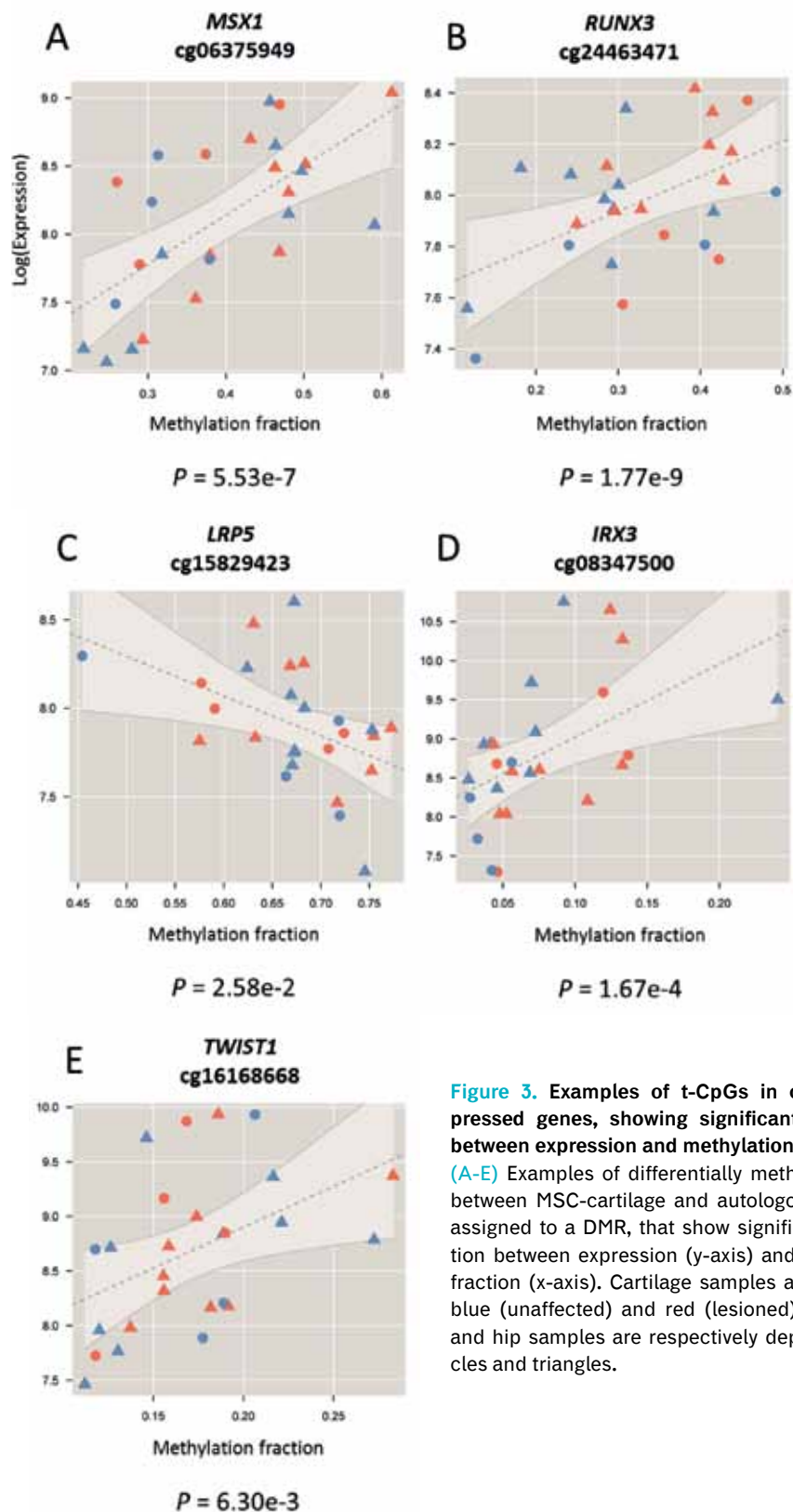


Figure 3. Examples of t-CpGs in cartilage expressed genes, showing significant correlation between expression and methylation.

(A-E) Examples of differentially methylated CpGs between MSC-cartilage and autologous cartilage, assigned to a DMR, that show significant correlation between expression (y-axis) and methylation fraction (x-axis). Cartilage samples are printed in blue (unaffected) and red (lesioned), while knee and hip samples are respectively depicted as circles and triangles.

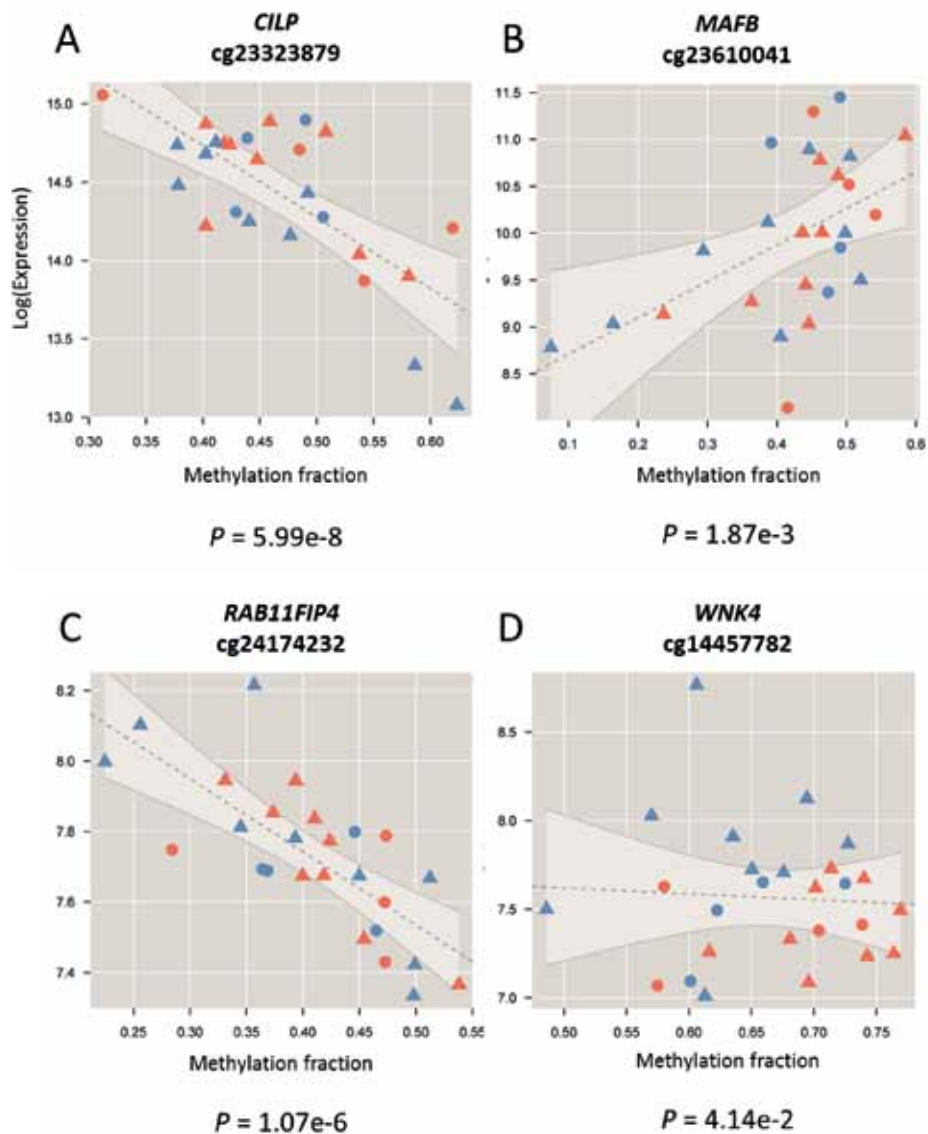


Figure 4. Examples of t-CpGs in cartilage expressed genes, showing significant correlation between expression and methylation.

(A-D) Examples of differentially methylated CpGs between PAC-cartilage and autologous cartilage, assigned to a DMR, that show significant correlation between expression (y-axis) and methylation fraction (x-axis). Cartilage samples are printed in blue (unaffected) and red (lesioned), while knee and hip samples are respectively depicted as circles and triangles.

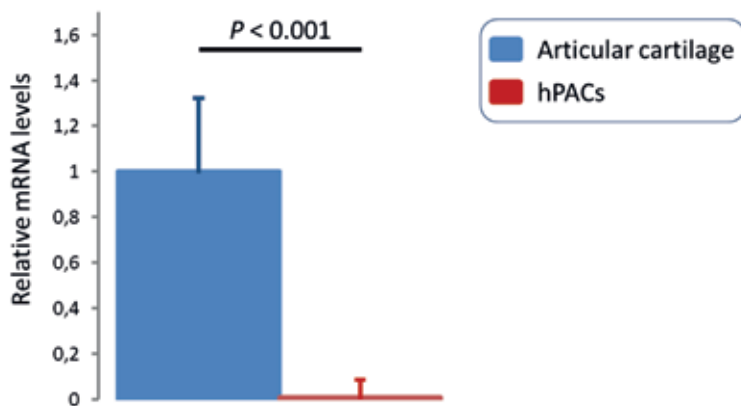


Figure 5. RT-qPCR analysis of *CILP* expression in in vitro cultured hPACs and autologous articular cartilage.

CILP mRNA levels were found down regulated 97-fold. Data is presented as the mean+SD of RT-qPCR triplicates and relative to articular cartilage expression.

pared to the chondrocytes directly isolated from the articular cartilage for DNA extraction (Figure 1), we identified CpGs that showed significant differential methylation ($P < 0.05$ & $\Delta\beta > 0.1$) between PAC-cartilage and the paired autologous, macroscopically unaffected, cartilage. As of such, we observed 5706 differentially methylated CpGs (1,33% of total CpGs) (Figure 2D; Table S5). Here, no significant differences in methylation were obtained as a consequence of timepoint of harvest. From the differential methylated CpGs, a total of 62 DMRs (45 hyper- and 17 hypo-methylated), comprising of 215 CpGs were called (Table S6). Because a single DMR can contain CpGs annotated to different genes these were mapped to 71 unique transcripts.

Pathway enrichment of all transcripts residing in these DMRs did not result in significant hits. Nevertheless, multiple DMRs were annotated to genes known to be involved in ECM composition (*CILP*, *COL9A3*, *SPON2* and *FBLN2*) and endochondral ossification (*GDF7*). Of these 8 CpGs (Table S7) were identified as t-CpG in the articular cartilage dataset as outlined above[35] and were annotated to 4 genes (*CILP*, *MAFB*, *RAB11FIP4* and *WNK4*) (Figure 4A-D). Except for *MAFB*, a negative correlation between DNA methylation and expression was observed.

Given the fact that *CILP* is amongst the highest expressed genes in articular cartilage[26], yet contains hyper-methylated t-CpG sites [35] in a DMR in PAC-cartilage as compared to autologous cartilage, we examined *CILP* expression levels in *in vitro* proliferated hPACs and analogous knee-cartilage tissue. A 97-fold decrease in expression in hPACs as compared to autologous cartilage (Figure 5) was observed.

Discussion

Here we show that chondrogenic 3D cultured MSC-cartilage exhibit an entirely distinct epigenetic signature as compared to 3D cultured PAC-cartilage and the respective autologous hip and knee cartilage. The epigenetic profile of PAC-cartilage, however, resembles 99% of that of the autologous cartilage from which the cells were isolated. This indicates that the use of hPACs for tissue engineering purposes could be favored above hBMSCs, despite the encountered difficulties with *in vitro* culturing, since the long term effects of the differential methylome are not known. In order to overcome this hurdle, the option of constructing neo-cartilage from hPACs, or even hPAC-derived iPS cells, instead of using hBMSCs as a cell source should be investigated[39].

Conversely, the effect of *ex vivo* culturing of hPACs showed only 1,33% of CpGs (5706) on the 450k array to be significant differentially methylated ($P < 0.05$ & $\Delta\beta > 0.1$). The resulting 62 DMRs were, amongst others, located near genes known to be involved in ECM homeostasis (*CILP*, *COL9A3*, *SPON2* and *FBLN2*) and endochondral ossification (*GDF7*). Furthermore, DMRs in 4 genes, *CILP*, *MAFB*, *RAB11FIP4* and *WNK4*, were identified to harbor t-CpGs that exhibit a significant correlation between DNA methylation and expression in articular cartilage[35]. We assessed the presence of t-CpGs, since these are believed to imply the direct relationship between the observed methylation and expression in articular cartilage. Nonetheless, our data shows that although methylation profiles at DMRs are highly tissue-type specific, they do not necessarily correlate to gene expression, as only a minority of DMRs appear to be associated with cartilage specific gene expression differences. Consequently, most of these CpG-methylation differences may therefore not be detected as t-CpGs in the study of *den Hollander et al.*[35] or vice versa.

The consistent positive correlation between methylation and expression, as seen for 4 out of 5 genes in figure 3, may not comply with the conventional inverse relation between CpG methylation and gene expression. However, in recent genome-wide approaches, it has been recognised that this conventional relation primarily holds among CpG dinucleotides residing in CpG islands and proximal promoters, whereas gene body and distal enhancer methylation, has been shown to correlate in either direction with gene expression[40-42]. For example, a recent study investigating the transcriptional associations of osteoarthritis-mediated loss of epigenetic control in articular cartilage showed that of 9,838 transcribed genes in articular cartilage, 2,324 correlated with the methylation status of 3,748 transcriptionally active CpG; both negative ($n=1,741$) and positive ($n=2,007$) correlations were observed[35].

Of particular interest was the finding of the transcriptionally active, hyper-methylation of a Cartilage Intermediate Layer Protein (*CILP*) annotated DMR ($\Delta\beta=0.16$) in PAC-cartilage, that corresponded to a profound decrease in *CILP* expression after *in vitro* culturing of hPACs as compared to autologous cartilage. Such a reduced expression of *CILP* during *in vitro* chondrogenesis of hPACs, is thought to increase the necessary *TGF- β 1*-induced *COL2A1* and *ACAN* expression; the key building blocks of articular cartilage and the formation of proper ECM. On the other hand, the extreme high levels of *CILP* expression in autologous articular cartilage[26], induced by *BMP2*[43], likely mark the relative

senescent state in which chondrocytes reside in healthy cartilage ECM. Although there is only a few percentage difference in the epigenetic landscapes of autologous cartilage and hPACs, these differences are likely markers for the dedifferentiation process which occurs when hPACs are brought into culture. Therefore we believe that the correlations as seen for CILP in this study, represent a transcriptional marker that is likely necessary for the formation of ex vivo neo-cartilage formation. As such, the effect of altered CILP expression on TGF-B1 induced anabolic processes and the corresponding consequence on integrity of the cartilage homeostasis warrants more in depth exploration, particularly for tissue regeneration purposes.”

To investigate whether the differences in the epigenetic landscape, observed between hBMSCs- and hPACs-derived and autologous cartilage, were actually driven by changes in the epigenetic landscape that occur during chondrogenesis both in MSC and PACs, we performed an analysis identifying CpGs with significantly methylation differences with time course ($t = 14, 21, 35$ and 49 days, and $t = 0, 4, 7, 14$ and 21 days respectively). As depicted in [Table S8](#), (1116 CpGs in hBMSCs) & [Table S9](#) (58 CpGs in hPACs), however, these CpG sites only slightly overlap with the differences in the epigenetic landscape observed between hBMSCs and hPACs derived and autologous cartilage shown in [Tables S3](#) (267 CpGs for hBMSCs) & [S5](#) (2 CpGs for hPACs). Ergo, it does not explain the large difference comparing autologous cartilage against hBMSCs and hPACs. It could be pointed out that the chondrogenically differentiated hBMSCs represent a mixed phenotype and that the differentiation is likely less advanced than the already differentiated hPACs. Nonetheless, we started to include hBMSC-derived micromasses from 14 days onwards, since our experience[27] showed that around this time point, the cells in the micromass cultures are differentiated in such a way, that they start producing cartilage-like extracellular matrix. At the more advanced timepoints of chondrogenesis (35 & 49 days of MSC-cartilage and 14 & 21 days of PAC-cartilage), the micromass cultures (deposited glycosaminoglycans) are optically similar in appearance ([Figure S1](#)) and stratified analyses of these late timepoints were found to display comparable epigenetic differences towards autologous cartilage as in the initial comparison.

Since the differences in DNA methylation between MSC-cartilage and autologous cartilage are substantial, the question should be raised whether the use of hBMSCs for tissue regeneration purposes is eligible. For example, DMRs that harbor CpGs with large methylation differences were found to be annotated to genes that are not only important for development, but are also associated with cancer pathways (*RUNX3*, *TWIST1*, *GLI3*, *WNT5A*, *TP63* and *STAT3*)[44-49]. The alternative epigenetic regulation found in MSC-cartilage is therefore difficult to explain and we do not yet know what the long term consequences are of implanting regenerated tissue that is epigenetically very distinct from its target tissue. It was reviewed recently, that results from pre-clinical studies suggest that the MSCs injected for regeneration purposes served an directive role as opposed to supplying the direct building blocks[50].

Given that after *in vitro* proliferation for 2 passages and 3D chondrogenic culturing, the methylome of hPACs still resembles almost 99% of that of autologous cartilage, the utilization of hPACs, instead of hBMSCs for tissue regeneration purposes, should be seri-

ously considered. In light of the increasing proof of the generic presence of adult tissue-specific stem cells[51], it could also be hypothesized that by isolating and proliferating primary chondrocytes, we enrich the cell culture for highly specialized, unipotent adult cartilage stem cells. This cell population could in theory be, epigenetically, perfectly programmed for chondrogenesis purposes. A first step to substantiate this hypothesis is to search for such cartilage resident adult stem cells

Here we have shown that, based on genome wide DNA methylation profiles, neo-cartilage engineered from hPACs is almost identical to its autologous donor. In contrast, neo-cartilage engineered from hBMSCs, has a distinct DNA methylation profile as compared to autologous cartilage. Many transcription factors important for general development and morphogenesis were shown to harbor DMRs. So, although hBMSCs are widely used for cartilage engineering purposes, the effects of these vast methylation differences on cartilage regeneration and long term consequences of implantation, are not known. Therefore, the choice of cell source for future cartilage tissue regeneration purposes should be investigated in future endeavors.

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Competing interest

The authors declare no competing financial, personal, or professional interests.

Author contributions

Conceived and designed the experiments: NB, IM.

Performed the experiments: NB; HS; EH; YR

Acquisition of material and data: NB, WH, RS, HS, RN, YR.

Preparation of the manuscript: NB, IM.

Critical reviewing and approval of the manuscript: all authors.

Supplemental Data

[http://www.oarsijournal.com/article/S1063-4584\(16\)01070-0/addons](http://www.oarsijournal.com/article/S1063-4584(16)01070-0/addons)

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Chapter 7

Summary and general discussion

Parts of this text are published as a review:

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Introduction

In the field of OA research the step from genetics to biological functionality, also named ‘functional genomics’, is necessary to allow valorisation of genetic findings¹⁻³, thereby augmenting the need for functional data of disease relevant tissues. Even so, it was estimated that pursuing druggable targets directed by genetic studies are twice as often successful as compared to those without it⁴. In this thesis we apply the functional genomics methodology, to proceed from a genetic association to mechanistic understanding of the effect of genetic variation on gene expression and epigenetic regulation contributing to OA susceptibility. Particularly we set out to characterize and validate the pathophysiological processes that underlie the role of *DIO2*/thyroid hormone signalling in the onset of OA after identifying the *DIO2* gene as a OA susceptibility locus. We hypothesized that upregulation of *DIO2* and thereby increased thyroid hormone signalling may be key in the etiology of OA, whereas mitigating *DIO2* expression could possibly prolong cartilage homeostasis. For that matter, disease specific tissue analysis, customized cell- and tailored mouse-models, were applied. Proper organisation and expansion of a disease-specific tissue- and cell-bank (RAAK) was executed as part of this thesis and resulted in a tailored toolbox, ideal to dissect molecular mechanisms underlying disease onset. Our progress in functional genomics was supported by this collection of cartilage tissue samples in a large number of OA patients in a design allowing for patient specific comparison of preserved and OA affected cartilage. Human bonemarrow-derived mesenchymal stem cells (hBM-MSCs) and human primary articular chondrocytes (hPACs) of multiple donors were eligibly subjected to *in vitro* micromass culture experiments, which allowed for functional validation and causal inference of identified susceptibility genes.

Translating genomics into mechanisms of disease; functional genomics in OA

A first important step from genetic susceptibility to underlying disease mechanism is understanding the functional consequences of the identified risk allele. The effectiveness of assessing allelic imbalanced expression (AIE), in OA research was shown previously for *DIO2*⁵. In the current thesis AIE was implemented for the identified hand OA susceptibility gene *ALDH1A2* outlined in [chapter 2](#). Besides assessing differential *ALDH1A2* expression levels in OA effected cartilage as compared to preserved cartilage isolated from the same joint, we provided proof for an allelic imbalance in expression of the gene, dependent on the genotypes of the rs3204689 SNP in the 3’UTR of the *ALDH1A2* transcript. We showed that the transcript with the risk-conferring C allele was expressed at a lower level than the corresponding normal transcript. Our expression data supported a role for *ALDH1A2* in mediating the effect of the osteoarthritis risk variants in the associated locus, indicating likely causality for the genetic variant. Nevertheless, to understand the effect of the identified AIE and differential gene expression, further

research is needed to dissect molecular effect on articular cartilage. The use of tailored cell-models to do so is exemplified in this thesis for *DIO2*.

Functional genomics of *DIO2*; intracellular thyroid hormone signalling

DIO2 expression and DNA methylation in disease specific tissues

As mentioned before, *DIO2* expression was shown absent in healthy cartilage, but was found highly upregulated in both macroscopically normal and lesioned OA cartilage⁶⁻⁸. Carriers of the *DIO2* rs225014 risk allele ([chapter 3](#)) were found to have increased *DIO2* gene expression in affected- compared to preserved cartilage. A consistent positive correlation was further demonstrated between methylation at the CpG site 2031 base pairs upstream of the *DIO2* transcription start site (CpG-2031) and *DIO2* expression in articular cartilage among carriers of the rs225014 risk allele. These regulatory properties of DNA methylation on *DIO2* expression was subsequently confirmed by applying 5-aza-2'-deoxycytidine (AZA) treatment eliciting general demethylation of the DNA, concomitant with a decrease in methylation at CpG -2031 and *DIO2* expression⁹. Given that the CpG-2031 maps within an active CCCTC-transcription factor (CTCF) binding site, we hypothesized that methylation dependent binding of CTCF could act as a positional isolator of *DIO2* expression⁹. Albeit that we found partial proof for this hypothesis, we were not able to confirm three-dimensional chromatin conformations to underlie the relation between the rs225014 tagged allelic imbalance and methylation-dependent upregulation of *DIO2* among rs225014 risk-allele carriers. Nonetheless, our data provided evidence in humans that genetic predisposition combined with OA-related changes of the articular cartilage resulted in loss of epigenetic silencing of *DIO2*. The re-initiated expression of *DIO2* in adult OA affected articular cartilage due to the increase of DNA methylation could be part of the observed unfavourable recuperation of early life signalling of chondrocytes in OA affected cartilage^{10, 11}. The fact that the presence of increased *DIO2* expression in preserved cartilage by itself does not initiate OA, shows that a secondary trigger is needed. Since OA lesions are predominantly found at the loading 'hotspots' of the OA-joints, it was hypothesised that high *DIO2* expression combined with biomechanical (over-)loading could be the combined trigger to commence cartilage degradation marking the onset of OA. This hypothesis was further addressed in [chapter 4](#).

DIO2 perturbation in customised in vitro chondrogenesis models

Furthermore in [chapter 3](#), the *in vitro* micromass chondrogenesis model of human bone-marrow derived mesenchymal stem cells (hBM-MSCs), was applied to investigate the direct effect of *DIO2* upregulation on cartilage homeostasis and integrity in the development of human OA. We showed that in this OA model, upregulation of *DIO2* expression as well as subsequent increased T3 bio-availability, was sufficient to directly induce detrimental expression of the OA catabolic markers of cartilage destruction. A marked reduction of the chondrogenic capacity of the cells to deposit ECM components was found, concur-

rent with induction of OA specific markers of cartilage matrix degeneration (*ADAMTS5* and *MMP13*) and mineralization (*ALPL*). Given their concurrent upregulation, it was hypothesized that this process is likely mediated via *HIF-2 α /RUNX2* signalling, an important pathway in the OA disease process. ChIP-seq data covering thyroid hormone receptor (THR) binding supported this hypothesis by showing that the *HIF-2 α* locus harbours specific THR binding sites and is directly transcriptionally reactive to thyroid hormone¹². In view of these data, we advocate that active thyroid hormone availability, likely by local *DIO2* action, in the *in vitro* OA-model of hBM-MSCs has an important impact on *HIF-2 α* upregulation, a hallmark in the pathophysiology of OA.

Although we found a direct detrimental effect of increased *DIO2* expression and subsequent T3 availability on matrix deposition in this *in vitro* model, this finding was not in line with the observations made earlier in OA and preserved cartilage tissue. Preserved cartilage was shown to express high levels of *DIO2*, similar to OA affected cartilage, nevertheless being macroscopically unaffected. Additionally, we found that increased T3 bioavailability did not have a similar profound, detrimental effect on cartilage explant cultures, nor on *in vitro* 3D micromass cultures of primary chondrocytes, both isolated from preserved regions of the OA joints (unpublished data). In line with this, cartilage-specific h*DIO2* overexpressing (hD2Tg) rats did not show any inherent defects on the femoral or tibial articular cartilage surfaces¹³. However, submitting the hD2Tg rats to an injury-induced OA model (DMM), thereby applying an acute biomechanical burden on articular cartilage, resulted in more cartilage damage as compared with their wild-type littermates¹³. These findings, again, indicated that although *DIO2* is upregulated in an OA affected joint, such chondrocytes are not necessarily in an OA like state and additional perturbation is necessary to initiate terminal maturation and irreversible breakdown. With respect to the distinctive feature of BM-MSCs, to undergo all subsequent steps of the endochondral ossification program, ending in terminal maturation and apoptosis of the chondrocyte, we advocate that the tendency of these cells to commence a growth plate chondrocyte-like identity, BM-MSCs are ideal for pathophysiological OA research. Even though hBM-MSCs are mechanistically prone to do so, intervention with the general deiodinase inhibitor, Iopanoic Acid (IOP), contributed to prolonged ‘healthy’ cartilage homeostasis by virtue of attenuated upregulation of matrix degrading enzymes, a constant *COL2A1/COL1A1* ratio, denser cartilage matrix structure with significant less cellular lacunae, thereby indicating a reduced propensity of chondrogenically differentiated hBM-MSCs to enter the terminal maturational process. This suggested that in a fully activated OA process of endochondral ossification the deiodinase inhibitor IOP was able to arrest terminal maturation. These findings showed promising results for future therapeutic intervention, regarding thyroid hormone signalling and were encouraging further research in an *in vivo* model.

***DIO2* deficiency in tailored *in vivo* animal models; Exercise induced OA**

The beneficial effect of inhibiting *DIO2* was translated to a mouse model in [chapter 4](#), in collaboration with the Skeletal Biology and Engineering Research Centre (KU Leuven,

Belgium). We assessed the effect of forced exercise, in a treadmill running model of OA, on knee-joints in *Dio2*^{-/-}-mice and their wild-type littermates. No inherent histological differences were found between articular cartilage of *Dio2*^{-/-}-mice and their wild-type littermates. Nonetheless, we showed that *Dio2*^{-/-}-mice were protected against exercise induced OA as compared to wild-type-mice. Upon forced mechanical loading, only the wild-type-mice showed clear signs of cartilage damage and synovitis, supporting the use of forced running as an OA-model in mice. Biomechanical stress resulted in 147 transcripts to be significantly differentially expressed between the challenged (running) and unchallenged (non-running) mice in a genome wide expression analysis. However, cartilage from *Dio2*^{-/-}-mice was found to exhibit significant stress-induced expression. This absence and the non-appearance of histological cartilage damage in *Dio2*^{-/-} mice suggested that degenerative pathways were not activated in this knockout strain despite the biomechanical burden imposed. Moreover, we found that *Dio2*-deficiency was reflected in a specific expression profile of genes showing significant interaction between *Dio2*-genotype and biomechanical stress that either mark a favourable effect in *Dio2* knockout- (eg. *Gnas*) or an unfavourable effect in wild-type-cartilage homeostasis (eg. *Hmbg2* and *Calr*).

How histologically similar cartilage of *Dio2*-deficient, as compared to wild-type mice, was able to withstand the detrimental effects of mechanical stress is not clear. It was brought forward that the effects of systemic *Dio2*-deficiency could have an impact on general metabolism of these mice, and thereby regulating the effect as seen in the OA model. Of note here, it was shown that *Dio2*-deficient mice had a normal circulating T3 concentration as compared to wild-type-mice¹⁴, disqualifying the effect to be attributed to systemic alteration of circulating T3. Furthermore, results presented in a concurrently supporting paper, correspondingly showed that articular cartilage of *Dio2*-knockout as compared to wild type littermates did not have intrinsic histological alterations in joint cartilage, nor in subchondral bone area¹⁵. This again showed, like for the hD2Tg rats, that aberrant *DIO2* expression by itself does not initiate OA-like macroscopical changes in the articular cartilage. Nonetheless, mineral content of the subchondral bone was found to be markedly increased in *Dio2* knockout-mice, resulting in bones with reduced toughness and increased susceptibility to fracture. How this bone abnormality in *Dio2*-deficient mice reflects its role in OA pathogenesis is unknown and it stays unclear whether alterations in subchondral bone and articular cartilage occur simultaneously or whether subchondral bone changes result in cartilage damage or vice versa^{15, 16}. Overall, these findings further support the hypothesis that the combination of *DIO2* expression and mechanical stress is essential to commence OA. Furthermore, it provided additional *in vivo* support that interfering with intracellular thyroid hormone levels could be a powerful way to oppose the pathological events that are occurring in OA as a result of biomechanical burden, in particular since no striking developmental skeletal phenotype appears present in the *Dio2*^{-/-}-mice.

Intracellular thyroid hormone; a master switch to joint destruction?

In summary, articular chondrocytes may lose their healthy maturational arrested state

throughout life as a result of loss of epigenetic regulation due to mechanical stress, injury or age and convert into a hypertrophic state as reflected by up regulation of *DIO2* in preserved and lesioned OA articular cartilage^{6, 7, 17, 18}. By applying functional follow up studies to the OA susceptibility gene *DIO2*¹⁹ (Box 1) it was demonstrated that aberrant intracellular T3 signalling, likely modified by genetic variations in recognised OA susceptibility genes such as *DIO2*, *NCOA3*²⁰, *ALDH1A2*²¹, *SMAD3*²² and *DIO3*²³, disturbs adult articular cartilage homeostasis and provokes irreversible articular cartilage damage, particularly upon external stresses (Figure 1). These results showed that intracellular thyroid hormone signaling could be underlying a more common OA, and we therefore hypothesize that the induction of intracellular thyroid hormone signalling may be a master-switch that forces hypertrophic chondrocytes to initiate terminal maturation and inflict irreversible articular cartilage damage upon external stresses. Moreover, upon exploring ways to counteract the aberrant *DIO2* effect it was indicated that local T3 signaling could be mitigated by IOP. The data in this manuscript builds on a dedicated ongoing functional genomic OA research-line. By making use of the functional genomics pipeline, we²⁴⁻²⁷ and others^{6, 28} have provided mechanistic insight in the role of *DIO2*¹⁹ in OA and provided an evidence based treatment option. These insights strongly suggest that particularly intracellular levels of thyroid hormone should be controlled tightly, especially under biomechanical perturbations, to maintain joint tissue homeostasis.

Timeline for DIO2 and Osteoarthritis; from genetic susceptibility to functional genomics

2008	Identification of <i>DIO2</i> (rs225014) as an OA susceptibility locus ¹⁹ .
2008-2010	upregulated <i>DIO2</i> mRNA levels with disease progression ^{6, 7, 17, 18} .
2012	Increased <i>DIO2</i> protein in OA affected cartilage and allelic imbalance of OA risk SNP (rs225014) in OA tissues ²⁵ .
2013	<i>DIO2</i> upregulation causes cartilage destruction in an OA-rat model ²⁸ .
2014	Identification of underlying (epigenetic and transcriptomic) mechanisms of <i>DIO2</i> susceptibility in OA ²⁴ .
2014	Attenuating D2 activity with IOP results in prolonged 'healthy' cartilage homeostasis ²⁴ .
2014	Knocking out <i>Dio2</i> in a murine model protects against cartilage damage only upon biomechanical burden ^{26, 29} .

Intrinsic factors for healthy cartilage; *DIO2* deficiency

In parallel to OA disease modifiers, there is a need for insight into intrinsic factors that enhance stability of healthy articular chondrocytes also upon environmental challenges such as biomechanical (over-) loading. In chapter 5, we focused on *Calreticulin* (*Calr*), the single transcript reaching the significance threshold when comparing cartilage of wild-type- and the protected *Dio2*^{-/-}-mice. Notably, our previous data showed that biome-

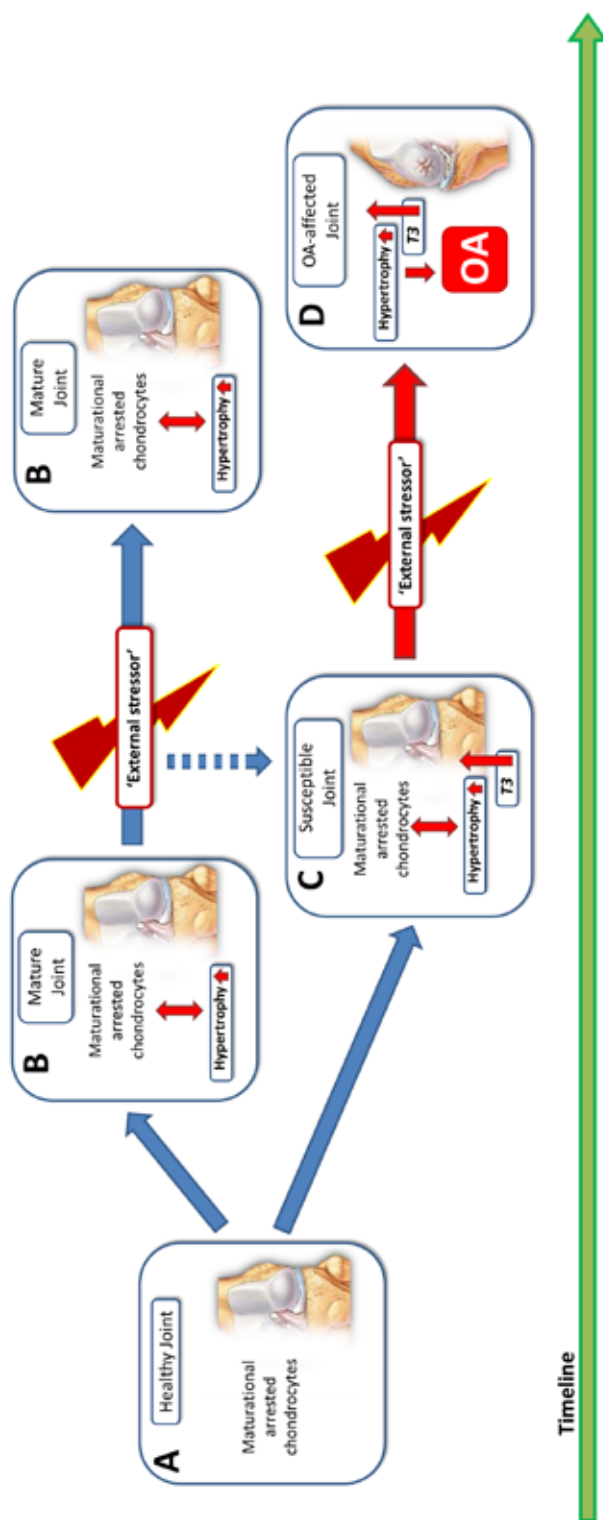


Figure 1: Graphical representation of hypothesized importance of thyroid hormone in the onset of OA. (A) In healthy joints, chondrocytes reside in the articular cartilage in a maturational arrested state. With ageing, chondrocytes of the adult joint can become hypertrophic (B) but this is hypothesised not to be crucial for the onset of OA. External stressors, like injury and joint damage, applied on these joint

are not necessarily the tipping point towards irreversible cartilage damage, but can make the joint susceptible for OA. (C) The additional re-initiated bioavailability of active thyroid hormone, in these susceptible joints, is here proposed as the master-switch that initiates irreversible cartilage damage under the influence of mechanical stress (D) likely due to terminal maturation of the articular chondrocyte calcification of the matrix.

chanical stress had a profound effect on *Calr* expression in cartilage of wild-type-mice, whereas *Calr* expression was not altered in *Dio2*-knockout-mice upon challenge. However, independent of challenge, *Calr* was found significantly downregulated in cartilage of *Dio2*^{-/-} mice and therewith accompanying the beneficial homeostatic state of the articular cartilage. We demonstrated that overexpression of *Calr* during early chondrogenesis lead to decreased proteoglycan deposition and corresponding lower Aggrecan expression. Knocking down *Calr* expression did not result in histological differences of matrix composition. In addition to this, expression levels of *Calr* and *Dio2* were found to be correlated. These data implicated that both lower levels of *Calr* and *Dio2* appear beneficial to cartilage homeostasis also upon mechanical perturbation.

The hypothesis on the underlying mechanism of *Calr*, resulting in the beneficial homeostatic state of articular cartilage, comes in twofold. First, by regulating the amount of calcium stored in the endoplasmatic reticulum, and therefore the amount that can be released to the cytosol to trigger downstream events, *Calr* could affect apoptotic outcomes³⁰. Given our data, we could hypothesize that lower levels of *Calr* expression, as a result of *Dio2*-deficiency, resulted in a more subtle calcium-induced apoptotic signal upon stress, being favourable for the maintenance of cartilage tissue homeostasis. The beneficial effect of reduced calcium-levels for cartilage integrity was supported by a recent study which reported that calcium antagonists might be efficient in preventing progression of OA³¹. Second, *Calr* was shown to interact with nuclear hormone receptors, like the glucocorticoid, androgen and retinoic acid receptor. Although interaction with thyroid hormone receptors was not yet shown, it was made presumable, since the gene family of nuclear receptors was characterized by the presence of a typical, well conserved DNA-binding domain³². This implicated that *Calr*, possibly interacts with thyroid hormone receptor initiated transcription and thereby interfering with activation of detrimental pathways, like *HIF-2α* signalling upon increased T3 bioavailability. This showed the opportunities of *Calr* as a therapeutic target in parallel to *DIO2*, interfering in or as a result of the thyroid hormone signalling cascade, in the treatment of OA.

Functional genomics; *in vitro* 3D cell models compared to autologous cartilage

Being triggered by the difference in effect of T3 bioavailability on the chondrogenic capacity of hBM-MSCs and hPACs, we focussed in [chapter 6](#) on the epigenetic landscapes of our *in vitro* micro-mass chondrogenesis models of hBM-MSCs and hPACs as compared to paired autologous articular cartilage. As discussed before, increased T3 bioavailability did not have a similar profound, detrimental effect on cartilage explant cultures, nor on *in vitro* 3D hPAC micromass cultures, both isolated from preserved regions of the OA joints, as in 3D hBM-MSCs micromass cultures. We suspected that this was due to an aberrant methylome in hBM-MSCs, making them more prone to commence OA initiating transcription. However, the exact underlying molecular mechanism is not understood and studies which explore comprehensive pathways in endochondral differentiation of

MSCs and chondrocytes had not been published in recent years³³. Our study showed that *in vitro* engineered neo-cartilage tissue from hPACs, exhibited a DNA methylation landscape that is almost identical (99% similarity) to autologous cartilage, in contrast to neo-cartilage engineered from hBM-MSCs. This showed that even though the model of hBM-MSC micromass cultures resembled a model of OA development³⁴, the cells in this neo-cartilage did not represent adult articular chondrocytes, that reside in macroscopically normal articular cartilage, based on their epigenetic signature, nor did they represent the shift towards OA affected chondrocytes (data not shown). Of note, the vast methylation difference was ruled out to be a result of the time course of chondrogenesis, since only a minority of CpGs showed significant methylation changes over time in both hBM-MSCs (0.25% of all CpGs) and hPACs (0.06% of all CpGs). Moreover, the majority of these time course-dependant CpGs did not contribute to the difference with autologous cartilage. Having said all this, as to date, hBM-MSCs are widely used for cartilage engineering purposes, even though the effects of these vast methylation differences on cartilage regeneration and long term consequences of implantation, are not known.

Our results suggested that the use of hPAC micromass cultures or autologous cartilage explant cultures should be utilized for OA research, since they displayed a more natural reflection of the autologous cartilage than the hBM-MSCs model. It is yet unclear, and unlikely, whether the processes as seen with OA occur spontaneous in this model. An external, though unnatural, stressor will be needed, such as e.g. biomechanical stress, predisposing the model for OA. Nonetheless, since an external stressor will be needed to commence onset of OA-like damage, the choice of external stressor used to imply this damage will influence the type of OA that is being investigated. For example, using IL-1 β will lead to an inflammation induced OA, as will the use of reactive oxygen species lead to cellular stress induced OA. The same holds up for the choice of mouse-model for research on the onset and development of OA, or the development of new drugs for that matter. The widely used DMM-model will help to understand the changes occurring in post-traumatic articular cartilage, as will the collagenase-induced model explain the inflammatory-induced OA. The model of mechanical loading by forced exercise³⁵ was put forward to provide a more physiological model of disease induction and progression³⁶. Nonetheless, we should always keep in mind that these models are not natural and strain the development of OA-like cartilage damage.

Future perspectives

In recent years accumulating evidence showed that counteracting the deleterious effect of *DIO2* by attenuation of thyroid signalling may be key in securing joint tissue homeostasis irrespective of the OA disease subtype. Future endeavours should be designed to demonstrate that local inhibition of D2 by intra-articular admission of IOP, could be an effective therapy to alleviate the burden of OA thereby increasing mobility, well-being and quality of life particularly among elderly. The reoccurrence of developmental processes in adult cartilage exemplifies the dysdifferentiation theory of ageing³⁷; methylation

changes cause transcriptional alterations with age, resulting in de-repression of genes in tissues where they are normally not expressed.

The availability of diseased, but unaffected cartilage explant-tissue allows us to establish a human *ex vivo* OA-model wherein different strains of mechanical loading induces different grades of OA-like cartilage destruction. It is generally accepted that biomechanical loading is necessary for the maintenance of cartilage homeostasis^{38, 39}. However, abnormal, altered or injurious loading is associated with inflammatory and metabolic imbalances that may eventually lead to OA like damage³⁹. Moreover, *ex vivo* cartilage explants subjected to these magnitudes of stress exhibit a significant suppression of metabolic activity and particularly affects the biosynthesis of aggrecan and collagen⁴⁰ similar to the *in vivo* situation²⁴. This model is ideal to assess the ability of compelling novel druggable targets, such as IOP, to prevent, stop or reverse OA. Furthermore, by applying this *ex vivo* “bench-work” it will be relatively easy to translate effect to *in vivo* models and even to human pre-clinical trials comprising patients with OA. Gaining knowledge on how biomechanical stress and injury provokes intracellular thyroid hormone signalling in articular joints to bring about its detrimental effects in mature articular cartilage is essential to understand the mechanisms for future therapy. Given that *DIO2* does not affect circulating thyroid hormone levels in humans²⁴, these investigations should focus on and target the local adaptation to T3 availability of the articular cartilage. Testing the therapeutic effects of deiodinase inhibitor IOP, *ex vivo* and *in vivo*, will give us valuable information on the potential clinical use. Nevertheless, we need to keep notice of the fact that we are looking for molecular explanations for OA onset and development in artificial models, both *in vitro* and *in vivo*. Therefore our findings should always be placed in perspective and, like in the discovery and interpretation of genetic- and bio-markers, characterize OA-subtypes in the different *in vitro* and *in vivo* models that are used to dissect underlying molecular mechanisms^{36, 41, 42}.

Results of the studies performed during this PhD have led to the translational research proposal, supported by the Dutch Arthritis Foundation, in which the beneficial effects of attenuated *DIO2* expression by means of intra-articular IOP injection will be investigated on cartilage integrity in both *ex vivo* explant- and *in vivo* mouse-models of OA.

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Chapter 8

Nederlandse samenvatting

Inleiding

Osteoartrose, in de volksmond kortweg artrose genoemd, is de meest voorkomende gewrichtsaandoening en wordt gekenmerkt door pijn en stijfheid van de gewrichten, met als gevolg chronische invaliditeit. De oorzaak van artrose is de toenemende afbraak van kraakbeen en de vorming van botuitstulpingen aan de randen van de gewrichten (osteo-phyten). Deze symptomen kunnen voorkomen in alle gewrichten van het lichaam, maar uiten zich voornamelijk in hand-, heup- en knie- gewrichten.

Artrose wordt beschouwd als een zogenaamde complexe ziekte. Dit houdt in dat het ontstaan van de ziekte een samenkomst is van genetische- en omgevingsfactoren, zoals (sport) blessures, overgewicht, geslacht en leeftijd. Doordat zo veel verschillende factoren van invloed zijn op het ontstaan en het verloop van de ziekte is de biologische oorzaak nog grotendeels onbekend. Dit heeft als gevolg dat tot op heden de behandelmogelijkheden voor artrose beperkt zijn tot het geven van pijnverlichting totdat tenslotte een gewrichts- vervangende operatie noodzakelijk is.

Genetisch onderzoek heeft in de loop der jaren al wel geleid tot de ontdekking van een aantal risicogenen voor artrose, zoals *DIO2*, *GDF5*, *DOT1L*, *NCOA3* en *SMAD3*. Echter, om te komen tot de biologische werking van deze risicogenen en de waarde van een genetische bevinding in te schatten zijn functionele studies vereist. Om deze stap te kunnen maken moet je de beschikking hebben over ziekterelevante weefsels, waaronder dus kraakbeen. Belangrijk hierbij is ook om je te realiseren dat dat medicijnonderzoek, ondersteund door genetisch bewijs, twee keer zo vaak succesvol is dan wanneer er geen genetische oorsprong is.

Doel van dit proefschrift

In dit proefschrift hebben we functionele studies gebruikt om van een genetische bevinding te komen tot een biologisch mechanisme dat leidt tot artrose. We hebben gekeken naar de effecten van genetische variatie op genexpressie en epigenetische regulatie en hoe dit bijdraagt aan de gevoeligheid voor artrose. Deze kennis kan gebruikt worden als aangrijpingspunt voor de ontwikkeling van een nieuw medicijn. Meer specifiek hebben we de ziekte processen ontrafeld die de rol van *DIO2*/schildklierhormoon in het ontstaan van artrose blootleggen. Genetische studies hebben laten zien dat erfelijke variatie in het *DIO2* gen risicoverhogend is voor het krijgen van de aandoening. Het *DIO2* gen codeert voor het enzym dat verantwoordelijk is voor de intracellulaire omzetting van inactief (T4) naar actief schildklierhormoon (T3). Van deze hormoon activatie weten we al dat het in de ontwikkelingsfase van het lichaam een belangrijke rol speelt in de vorming van de botten en gewrichten. Wat de precieze rol van verhoogde *DIO2* expressie en dus verhoogde T3 omzetting in een volgroeid gewricht is, weten we tot dit moment nog niet.

Voor het vinden van een verklaring en een mogelijke oplossing is het verkrijgen van ziekterelevante weefsels van essentieel belang. De verzameling hiervan, zoals gedaan voor een groot aantal artrosepatiënten in de RAAK-biobank, is ideaal gebleken voor het ontrafelen van moleculaire processen die onderliggend zijn aan het ontstaan van kraakbeenafbraak. Op deze manier konden we patiënt-specifieke vergelijkingen maken tussen

het aangedaan (artrotisch) en niet-aangedaan kraakbeenweefsel. Daarnaast hebben we als onderdeel van de RAAK-biobank mesenchymale stamcellen uit het beenmerg (hBM-MSCs) en primaire articulaire chondrocyten (hPACs) van meerdere artrosepatiënten verzameld. Met de cel modellen die we hebben ontwikkeld waren we in staat om reeds ontdekte risicogenen functioneel te valideren en, waar mogelijk, op in te grijpen. De juiste organisatie en uitbreiding van deze ziekterelevante weefsel- en celbank is uitgevoerd als onderdeel van mijn onderzoek.

In eerder onderzoek hadden we al verhoogde expressie van de risicovariant, ook wel risico allel genoemd, van *DIO2* ten opzichte van het referentie allel laten zien. Verder zagen we dat kraakbeen, aangetast door het artrose proces, meer *DIO2* mRNA en eiwit produceert dan gezond kraakbeen. Deze bevindingen lieten ons zien dat de genetische variant van het *DIO2* gen daadwerkelijk biologische gevolgen kan hebben voor het kraakbeen. Onze hypothese over de invloed van *DIO2* op het artrose proces luidde dat toegenomen expressie van *DIO2*, en daardoor toegenomen beschikbaarheid van T3, een bepalende factor is in de schadelijke processen onderliggend aan artrose. Omgekeerd veronderstelden we dat het remmen van deze *DIO2* expressie positieve gevolgen zou moeten hebben voor de gezondheid van het kraakbeen.

Hoofdstuk 1 geeft een inleiding over de aandoening artrose, het relevante weefsel en de reeds bekende ziekte mechanismen. Verder wordt er toegelicht welke methodologie er toegepast wordt in het artrose onderzoek en van welke ziekterelevante weefsels en specialistische cel- en muismodellen we in dit proefschrift gebruik hebben gemaakt om onze hypothese te bevestigen.

Van genetica naar ziekte mechanismen; functionele studies in artrose onderzoek

De ervaring heeft geleerd dat genetische variatie die de gevoeligheid voor het ontstaan van complexe ziekten verhogen vaak veroorzaakt wordt doordat het risico allel de expressie van een nabijgelegen gen beïnvloedt, bijvoorbeeld door alternatieve binding van transcriptie factoren.

Een eerste belangrijke stap om van genetische susceptibiliteit naar een onderliggend ziekte proces te komen, is te begrijpen wat de functionele consequenties zijn van het geïdentificeerde risico allel. Een methode om te bepalen of een variant de expressie beïnvloedt is via het meten van de hoeveelheid mRNA van de twee allelen afzonderlijk. Om dit onderscheid te maken tussen de twee allelen kun je weer gebruik maken van de genetische variatie die ook aanwezig is op het mRNA. De aanwezigheid van allelische onbalans, waarbij het risico allel kan dus meer of minder tot expressie komen in dragers van de variant dan de referentie, kan zo bijdragen aan het ontstaan van de aandoening. Het nut van het bepalen van allelische onbalans in expressie voor artrose onderzoek was reeds gedemonstreerd voor *DIO2*. In **hoofdstuk 2** gebruikten we deze methode om functioneel bewijs toe te voegen aan de identificatie van *ALDH1A2* als een risicogen voor hand artrose. Naast het identificeren van een verschil in *ALDH1A2* expressie tussen aangedaan en niet-aangedaan kraakbeen hebben we allelische onbalans aangetoond voor *ALDH1A2*. Deze bleek afhankelijk te zijn van dragerschap van de rs3204689 SNP in de 3'-UTR van het *ALDH1A2* mRNA. We hebben laten zien dat het mRNA met het risico allel "C" minder tot ex-

pressie kwam dan het corresponderende referentie allel. Dit resultaat impliceert dat het risico allel een functioneel effect heeft op de expressie van het gen, maar om het exacte mechanisme te bepalen is verder onderzoek nodig. Voorbeelden van vervolgonderzoek zijn in dit proefschrift uitgevoerd voor *DIO2*.

Functionele studies van *DIO2*; de effecten van intracellulair schildklierhormoon

In hoofdstuk 3 gebruikten we gepaard aangedaan en niet-aangedaan kraakbeenweefsel om de veranderingen in expressie van het *DIO2* gen te bepalen. We lieten zien dat in aangedaan kraakbeen, dragers van het rs225014 risico allel een grotere toename van expressie van het *DIO2* gen laten zien dan niet-dragers. Daarnaast toonden we aan dat methylatie van een specifieke locatie net buiten het *DIO2* gen positief correleerde met *DIO2* expressie in dragers van de rs225014 variant. Met deze data hebben we aangetoond dat genetische aanleg samen met artrose gerelateerde veranderingen in het articulaire kraakbeen, resulteerden in een verlies van epigenetische onderdrukking van *DIO2* expressie. Vervolgens lieten we zien dat teveel expressie van het *DIO2* gen, en daarmee verhoogde aanwezigheid T3, slecht is voor de kwaliteit van het kraakbeen. In een *in vitro* chondrogenese model van hBM-MSCs leidde dit tot de activatie van ongunstige expressie profielen, resulterend in kraakbeen afbraak. Omgekeerd lieten we ook zien dat wanneer *DIO2* activiteit geremd wordt, dit een positief effect heeft op de gezondheid van kraakbeen. Deze bevindingen lieten zien dat een commercieel medicijn (Iopanoic acid; IOP), gericht op de remming van *DIO2*, de potentie heeft om gebruikt te worden als toekomstige interventie voor artrose.

Het positieve effect van de remming van *DIO2* expressie in *in vitro* kraakbeen hebben we vertaald naar een *in vivo* muismodel in hoofdstuk 4. In samenwerking met het 'Skeletal Biology and Engineering Research Centre' (KU Leuven, België) hebben we het effect onderzocht van *Dio2* op schade in muizenknieën veroorzaakt door mechanische overbelasting. We maakten gebruik van een model waarin muizen gedwongen werden om te rennen op een lopende band gedurende 3 weken (5 dagen per week, een uur per dag). Normale muizen hebben na deze zware belasting van de gewrichten duidelijke schade aan het kraakbeen. Echter, wanneer *Dio2* expressie afwezig is, leidt dit tot minder kraakbeenschade als gevolg van deze zware mechanische belasting. Genoomwijde expressie analyse liet daarnaast zien dat kraakbeen van wild-type muizen, onder invloed van de overbelasting, disregulatie van expressie van een groot netwerk van genen had. Echter, kraakbeen van muizen zonder *Dio2* ondervond hierdoor geen verandering op expressie niveau. De resultaten in dit hoofdstuk onderstrepen het belang van de *Dio2*-afhankelijke regulatie van T3 in de ontwikkeling van kraakbeenschade als gevolg van mechanische overbelasting.

Intrinsieke factoren voor gezond kraakbeen; *DIO2* deficiëntie

Naast het opsporen van factoren die het artrose ziekte proces beïnvloeden, is het ook belangrijk om meer inzicht te krijgen in intrinsieke factoren die bijdragen aan de stabiliteit van gezonde kraakbeencellen. Met andere woorden; welke processen hebben een gunstige uitwerking op het kraakbeen, zelfs onder negatieve invloed van buitenaf, zoals mechanische (over-)belasting? In hoofdstuk 5 hebben we ons gericht op *Calreticulin*

(*Calr*). Dit was het enige gen dat significant anders tot expressie kwam in kraakbeen van wild-type muizen vergeleken met muizen zonder *Dio2* expressie. Onafhankelijk van de mechanische belasting vonden we dat muizen zonder *Dio2* expressie een lagere expressie van het *Calr*-gen hebben dan de wild-type muizen. Daarnaast had mechanische belasting in de wild-type muizen een duidelijk verlagend effect op de expressie van *Calr*. Echter, in kraakbeen zonder *Dio2* was dit effect na overbelasting afwezig. Om de invloed van veranderende *Calr* expressie op de gezondheid van kraakbeen aan te tonen hebben we een *in vitro* celmodel gebruikt. Hierin lieten we zien dat verhoogde *Calr* expressie zorgt voor verminderde proteoglycaan synthese tijdens kraakbeen ontwikkeling. Proteoglycanen zijn belangrijke moleculen voor de integriteit van het kraakbeen. Het drastische verminderen van *Calr* expressie had daarentegen geen effect op de vorming van kraakbeenmatrix in vergelijking met de controle condities. De resultaten beschreven in hoofdstuk 5 laten zien lage expressie niveaus van zowel *Dio2* als van *Calr* bijdragen aan de gezond kraakbeen.

‘Functional genomics’; *in vitro* 3D-cel modellen vergeleken met autoloog kraakbeen

In hoofdstuk 6 hebben we ons verdiept in de vergelijking van het methylatie landschap van *in vitro* 3D-cel modellen met dat van gepaard autoloog kraakbeen. Aanleiding hiertoe was het waargenomen verschil in chondrogenese capaciteit van hBM-MSCs en hPACs als gevolg van de weefsel specifieke beschikbaarheid van T3. Onze studie liet zien dat *in vitro* gekweekt ‘neo-kraakbeen’ van hPACs een methylatie profiel had dat voor bijna 99% overeen kwam met dat van het oorspronkelijke kraakbeen. Dit in tegenstelling tot het neo-kraakbeen gekweekt van hBM-MSCs (~80% overeenkomst). Dus hoewel het *in vitro* 3D-model van hBM-MSCs een ideaal model is voor onderzoek naar het artrose ziekte proces, leken de cellen in het gevormde neo-kraakbeen niet op chondrocyten zoals aanwezig in kraakbeen van een volwassen persoon. Tenminste, op basis van hun methylatie profiel. Ondanks dit grote verschil in het methylatie profiel ten opzichte van normaal kraakbeen, worden hBM-MSCs wijdverbreid gebruikt voor weefsel regeneratie doeleinden. We weten echter niet wat het effect van deze abnormale regulatie is op de lange termijn na een transplantatie.

Conclusie en toekomstige toepassingen

De resultaten beschreven in dit proefschrift komen voort uit een toegewijde en voortgaande genomische artrose-onderzoekslijn. Door gebruik te maken van deze functionele studies hebben wij inzicht kunnen verkrijgen in de rol van schildklierhormoon gereguleerd via *DIO2* in het artrose proces. Het geleverde bewijs geeft een basis voor het ontwikkelen van een mogelijke behandeling. Onze resultaten doen vermoeden dat de intracellulaire T3 niveaus strikt gereguleerd moeten worden via *DIO2* als het kraakbeen mechanisch belast wordt, om stabiliteit van moleculaire processen in het kraakbeen te waarborgen. Het testen van de therapeutische effecten van IOP, *ex vivo* en *in vivo*, zullen ons waardevolle informatie leveren over het potentiële klinische gebruik ervan. Om een mogelijke effectieve behandeling voor artrose te vinden, zou toekomstig onderzoek zou gericht moeten zijn op de mogelijkheid om *DIO2* activiteit, en daarmee beschikbaarheid van T3, te verminderen met behulp van intra-articulaire toediening van een deiodinase-remmer (IOP) als.

Verder is het van essentieel belang om meer inzicht te krijgen in de mechanismen die in gewrichten zorgen voor de activatie van het T3-signaalsysteem onder invloed van bijvoorbeeld biomechanische stress. Gegeven dat *DIO2* geen invloed heeft op de hoeveelheid circulerend T3 in het lichaam, zullen deze onderzoeken zich vooral moeten richten op de lokale adaptatie van T3 beschikbaarheid in articulaire kraakbeen. Desalniettemin zullen we in ons achterhoofd moeten houden dat we op zoek zijn naar een moleculaire verklaring voor het ontstaan van artrose in een kunstmatig model. Daarom moeten we onze bevindingen altijd in perspectief plaatsen. Zoals het geval is bij genetisch- en biomarker-onderzoek, zouden we artrose-subtypen moeten karakteriseren in de verschillende *in vitro* en *in vivo* modellen die gebruikt worden.

Appendix

Curriculum Vitae

Nils Bömer was born Februari 1st, 1984 in Enschede, The Netherlands. He attended secondary school in Eibergen (first 3 years) and Haaksbergen (last 3 years) at the Assink Lyceum, where he passed his exams in 2002. In that same year he enrolled in his study Biomedical Engineering at the University of Twente, The Netherlands. He received his Bachelor of Science degree in 2005, after completed an internship at the department of Molecular Cell Biology and the BIOS Lab-on-a-chip chair at the University of Twente, where he looked at the use of microfluidic chips for single-cell electroporation. During the Master phase of his study he specialized by following the Molecular, Cellular and Tissue Engineering track. His first internship he performed at the Centro de Investigaciones Biológicas del Consejo Superior de Investigaciones Científicas (CIB - CSIC) in Madrid, Spain. He focused on developing cancer stem cell-specific monoclonal antibodies in mice at the Department of Cellular and Molecular Physiopathology of Prof. Dr. A. Silva. For his Master-thesis he did his internship at the department of Molecular Cell Biology at the University of Twente. Supervised by Prof. W. Kruijer and Dr. J.N. Post he assessed the influence of epigenetics on the expression of UTF-1, a stemness-state marker. In January 2010 he received his Master degree. With dedication and perseverance he applied for PhD projects where Molecular (Stem) Cell Biology, Epigenetics and the Clinic meet. In June 2011 he was appointed as PhD student at the department of Molecular Epidemiology under supervision of Prof. P. Slagboom and Dr. I. Meulenbelt. During his PhD he combined his three fields of interest to unravel the underlying molecular mechanisms of thyroid hormone signalling in the susceptibility to Osteoarthritis. The results of the research performed during the project are described and discussed in this thesis. From May 2016, he will be employed as a Post-doctoral researcher at the department of Experimental Cardiology of the UMCG, where he will study heart failure using functional genomics approaches.

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Presentations and grants

ORAL PRESENTATIONS

25th OARSI (OsteoArthritis Research Society International) conference

(April 30– May 3, 2015)

Concurrent session 4, Cartilage Biology

'Dio2-deficient mice are protected against cartilage damage in a model of exercise-induced OA'

Plenary session 4, Joint Homeostasis

'Dio2-knockout modulates circadian clock genes in articular cartilage through Thyroid hormone signaling'

Fourth annual meeting IDEAL (February 25-27, 2015)

Scientific progression

'Aberrant intracellular thyroid hormone signaling in articular cartilage: Master-switch for Osteoarthritis?'

1st MBE (Matrix Biology Europe) Conference (June 22, 2014)

Parallel workshop 3: Advances in understanding matrix disease mechanisms

'The effect of forced exercise on knee-joints of Dio2^{-/-} mice; identifying pathways involved in cartilage degradation processes following mechanical stress'

Third annual meeting IDEAL (February 26-28, 2014)

Scientific progression

'Underlying molecular mechanisms of DIO2 susceptibility in symptomatic osteoarthritis'

Presentation of Top 3 Competition Proposals

'Dietary intake is an important mechanism mediating OA susceptibility of the ageing cartilage'

Young Investigator Presentations

'Thyroid hormone effects on the chromatin modifiers expression is conserved between amphibians and humans'

First annual meeting IDEAL (March 1-3, 2012)

Scientific progression

'A human chondrogenesis model of mesenchymal stem cells Isolated from OA patients that underwent a joint Replacement surgery'

NVMB (Nederlandse Vereniging Matrix Biologie) conference (May 24 and 25, 2012)

'The functional effects of DIO2 in an in-vitro model for chondrogenesis'

GRANTS

Winner of the IDEAL young investigators competition 2014: €10.000

'Dietary intake is an important mechanism mediating OA susceptibility of the ageing cartilage'

Dutch Arthritis Foundation (translational research funding) 2015: €240.000

'Pharmacological attenuation of thyroid hormone signalling: An evidence based treatment option for Osteoarthritis' (T15-027) (supervised by I. Meulenbelt)

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List of abbreviations

AIE	Allelic imbalanced expression
ALDH1A2	Aldehyde dehydrogenase 1 family, member A2
AZA	5-aza-2'-deoxycytidine
BMI	Bodymass index
CALR	Calreticulin
ChIP	Chromatin immunoprecipitation
CILP	Cartilage intermediate layer protein
CpG	Cytosine-phosphate-Guanine dinucleotides
DIO2	Deiodinase iodothyronine type-2
DIO3	Deiodinase iodothyronine type-3
DIP	Distal interphalangeal joints
DMM	Destabilixation of the medial meniscus
DMR	Differentially methylated region
ECM	Extra cellular matrix
EMSA	Electrophoretic mobility shift assays
ER	Endoplasmatic reticulum
GAG	Glucoseaminoglycans
GWAS	Genome wide association studies
hBM-MSCs	Human bonemarrow-derived mesenchymal stem cells
hD2Tg	human DIO2 transgenic
hPACs	Human primary articular chondrocytes
IOP	Iopanoic acid
LD	Linkage disequilibrium
LMM	Linear mixed models
Mb	Mega base
MeDIP	Methylated DNA immunoprecipitation
MLR	Mean log expression ratio
OA	Osteoarthritis
OR	Odds ratio
PCA	Principal component analysis
PIP	Proximal interphalangeal joints
RA	Rheumatoid arthritis
RT-qPCR	Real time- quantitative polymerase chain reaction

RXR	Retinoid X receptor
SNP	Single nucleotide polymorphism
STRING-db	Search tool for the retrieval of interacting genes/proteins - database
T3	3,3',5-triiodothyronine
T4	3,3',5,5'-tetraiodothyronine
t-CpGs	Transcriptionally active CpGs
THR	Thyroid hormone receptor
TRE	Thyroid hormone response element
TTS	Transcriptional start sites
UTR	Untranslated region
SEM	Standard error of the mean

